Characterisation of Polycomb-group genes from *Antirrhinum majus* and *Arabidopsis thaliana*

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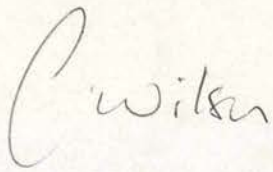
Declaration

I declare that this is my own work. Any contribution made by other parties is clearly acknowledged.



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Abbreviations

ATP	adenosine 5'-triphosphate
cDNA	complimentary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphosphates
EDTA	ethylene-diaminetetraacetic acid
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase
mRNA	messenger RNA
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
RT PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl-sulphate
Tris	tris(hydroxymethyl)aminomethane
µg	microgram
µl	microlitre
UV	ultra violet
X-gal	4-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-Glucuronide

Abstract

Polycomb-group (Pc-G) genes are a highly conserved group of transcriptional silencers that are believed to function by modifying chromatin structure. In animals they act to maintain the correct expression patterns of homeotic genes and many other targets during somatic development. In plants, several Pc-G genes with important developmental function have been characterised from the model organism *Arabidopsis thaliana*. The *CURLY LEAF* (*CLF*) gene, which shows homology with the *Drosophila* Pc-G gene *Enhancer of zeste* (*E(z)*), also regulates the expression of homeotic genes. In higher plants three classes of floral homeotic function exist, which act combinatorially to specify the identity of the four concentric whorls of the flower. *CLF* is required to repress the *c* function homeotic gene *AGAMOUS* (*AG*) in leaves, inflorescence stems and in outer floral whorls. Unlike the animal Pc-G genes, which act on many targets, the *Arabidopsis CLF* gene acts mainly on *AG*.

To test whether *CLF* homologues have a similar function in other plants, or have other targets, *CLF* homologues were isolated and characterised from *Antirrhinum majus*, a distant relative of *Arabidopsis*. Two genes were identified and named *ANTIRRHINUM CURLY LEAF 1* and *2* (*ANTCLF1* and *2*); both encode proteins that show greater similarity to CLF than any other plant Pc-G protein on databases. Because ANTCLF1 and 2 were more similar to one another than to CLF, it is likely that the genes arose from a recent gene duplication. Both genes are expressed within the meristem and primordia of vegetative and floral tissue consistent with a role during both vegetative and floral development. The strong similarity in sequence and expression suggests that the two genes may be functionally redundant. To determine their biological function, forward and reverse genetic screens were conducted to identify mutations that disrupt the genes, these proved unsuccessful.

Pc-G genes are thought to repress their targets in a heritable fashion. To investigate whether the *Arabidopsis* Pc-G gene *CLF* is required persistently to maintain *AG* repression, its time of action was investigated. A transgenic line with steroid inducible CLF+ activity was used to determine the developmental time of CLF silencing. A reporter gene for *AG* expression was introduced into this line to facilitate monitoring *AG* expression. Shifting experiments showed firstly that *CLF* is required persistently during development to repress *AG* in the leaves and inflorescence stem. Secondly, in leaves where *AG* is activated, restoring CLF+ activity was insufficient to repress *AG*. The implications for *CLF* action are discussed.

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1. Introduction

1.1 *Antirrhinum* as a model for the study of plant development

Several features make *Antirrhinum* a model plant for the genetic and molecular analysis of development. *Antirrhinum* is an annual with a relatively short generation time so that up to three generations can be grown in one year. The flowers of the *Antirrhinum* plant are bisexual and can be self-pollinated or cross-pollinated after emasculation. In addition the flowers are large (4-5 cm long) and therefore convenient for morphological analysis and the manipulation of pollination.

A key feature of *Antirrhinum* that has facilitated the isolation of many genes controlling developmental traits is that it possesses highly active transposable elements. Transposons are DNA segments with the ability to excise from one position in the genome and insert into another. Two families of TAM (Transposon *Antirrhinum majus*) elements have been identified based on sequence similarity and structural and functional similarities. The CACTA family includes TAM1, 2, 4, 5 and 6 which all have homologous terminal repeats. The second family contains the 3.5 kb TAM3 element which is related to Ac/Ds elements of maize.

The endogenous transposons of *Antirrhinum* have been utilised in forward genetic screens. Carpenter and Coen (1990) conducted a large-scale transposon-mutagenesis

experiment, in which TAM3 elements were mobilised. In screens of a large M_2 population consisting of 40,000 plants, numerous mutations were identified many of which were caused by transposon insertions. A number of floral homeotic mutations were identified, and the genes were isolated in a straightforward fashion as DNA flanking the transposon insertion. For example the meristem identity gene *FLORICAULA (FLO)* was tagged and cloned using this technique.

Transposon mutagenesis produced a floral homeotic mutation, designated *flo-613,* which had instability characteristics of Tam3 transposon induced alleles. Mutant plants grown at 15°C had occasional sectors of wild-type flowers, and these sometimes gave wild-type (revertant) progeny. Genomic DNA from *flo-613*, wild-type parental plants and revertant plants was digested with *Hind* III and hybridised using Tam3 sequences as a molecular probe. This revealed numerous bands, as Tam3 occurs in multiple copies in *Antirrhinum majus.* One band however was unique to *flo-613* plants; this 7.5 kb band was absent from wild-type progenitor and revertants. To clone this fragment a library was made from 7.5 kb fragments of *Hind* III cut DNA from *flo-613* plants. By screening the genomic library with a TAM3 probe, DNA flanking the insertion was obtained. This, in turn, was used as a probe to clone *flo* cDNA from a wild-type cDNA library (Coen *et al*, 1990).

Recently (e.g. Davies *et al*, 1999), transposon mutagenised populations have also been used for reverse-genetic screens, to identify insertions that disrupt a gene of interest. Unlike forward screens where no gene information is required, a genomic

or cDNA sequence of the desired gene is required for this method. It is particularly useful for the isolation of genes which may only have a subtle or no phenotype, for example genes where a phenotype is obscured by redundancy, since transposon insertion in the gene of interest is identified at the molecular level. In addition it can also be used to isolate genes whose mutant is homozygous lethal since the wild-type heterozygote can be identified. PCR amplification is extremely sensitive so it can detect a very rare template, for example DNA from a single mutant plant in a mixture of several thousand plants.

Antirrhinum provides a valuable comparative system for the study of plant development since the orthologues of many of the *Arabidopsis* floral homeotic *abc* function genes have also been characterised from *Antirrhinum.* This comparative system is highly informative since *Antirrhinum* is distantly related to *Arabidopsis*. In addition *Antirrhinum* has unique features that cannot be studied in *Arabidopsis.* The flowers of *Antirrhinum* are zygomorphic (bilaterally symmetrical) in comparison to *Arabidopsis*, which has actinomorphic (radially symmetrical) flowers. The interaction between floral homeotic genes and those controlling bilateral symmetry can therefore be analysed in *Antirrhinum* but not *Arabidopsis.*

1.2 Development of the aerial parts of *Arabidopsis* and *Antirrhinum*

Whereas in animals organogenesis occurs mainly during embryonic development, in flowering plants organ formation occurs continuously through the life of the plant, as a result of the activity of meristems. Meristems are located at the growing tips of the plant and consist of small regions of stem cells that undergo repeated divisions.

Two meristems initially develop from the plant embryo at either end of the apical-basal axis; these are the shoot apical meristem (SAM) and the root apical meristem. The SAM is responsible for the growth of the plant above ground. Cells at the top of the dome of the SAM divide and maintain it, whilst cells just below divide and differentiate to form the stem. On the periphery of the SAM dome specialised groups of cells divide to form either organ primordia or secondary meristems. The former produce leaves during the vegetative phase of growth. In *Antirrhinum* the SAM produces pairs of opposite leaves. Each pair is positioned at right angles to the next and these are separated by long internodes. In contrast *Arabidopsis thaliana* produces a spiral arrangement of leaves, which form a rosette during the vegetative stage of development, as the internodes are short and do not elongate (Figure 1.1).

Initiation of reproductive growth in the plant depends both on external and internal factors. Important external factors include temperature and day length. In *Antirrhinum* and *Arabidopsis,* long days (14-16 hours of light) promote the transition

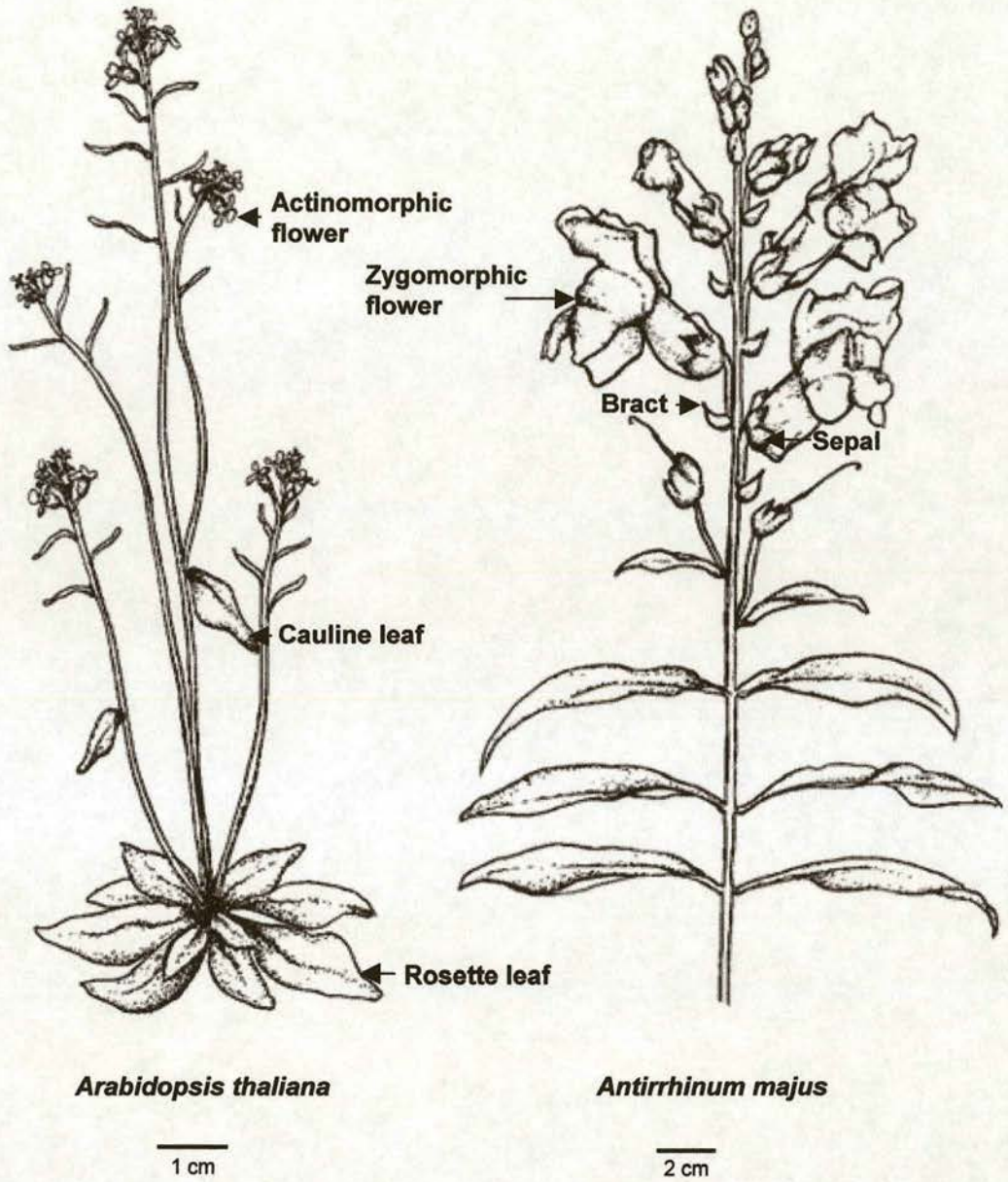

Figure 1.1 Wild-type *Arabidopsis thaliana* and *Antirrhinum majus* plants. *Arabidopsis* produces a rosette of leaves during vegetative development, the inflorescence of *Arabidopsis* is characterised by the production of cauline leaves and actinomorphic flowers. In *Antirrhinum* the shoot apical meristem produces pairs of opposite leaves, each pair of leaves is separated from the next by a long internode. The inflorescence of *Antirrhinum* produces very small leaf like bracts, flowers arise in the axils of these bracts. In contrast to *Arabidopsis,* the flowers of *Antirrhinum* are zygomorphic.

to reproductive development. This transition involves the transformation of vegetative shoot apical and lateral meristems into inflorescence meristems (IM's). The inflorescence meristem in turn produces a series of reproductive lateral meristems called floral meristems (FM's).

In *Antirrhinum* the inflorescence meristem produces very small leaf-like bracts in a spiral arrangement. Floral meristems are then initiated in the axils of these bracts (Figure 1.1). Two separate inflorescence phases have been identified in *Arabidopsis.* During the first inflorescence stage (I_1) a few small cauline leaves are produced in a spiral arrangement whereas in the second inflorescence stage (I_2) floral meristems are produced (Ratcliffe *et al*, 1999).

Both the vegetative and inflorescence meristems in *Antirrhinum* and *Arabidopsis* are indeterminate thus they can maintain their activity for a prolonged period of time and so produce an indefinite number of lateral organs and meristems. In contrast, floral meristems are determinate, they have a finite duration and produce a fixed number of floral organs. Carpel formation consumes the stems cells in the centre of the floral meristem and thus organogenesis ceases.

The floral meristem is initially a small dome shaped mound of cells that give rise to four concentric whorls of organ primordia. These primordia develop in highly stereotypical manner allowing distinct stages of early floral development to be

morphologically defined in a comparable manner in *Antirrhinum* (Carpenter *et al*, 1995) and *Arabidopsis* (Smyth *et al*, 1990; Table 1.1 below).

Table 1.1 Stages of early flower development in *Arabidopsis*

Modified from Smyth *et al*, 1990, Stages 1 to 5 are comparable with Stages 1 to 5 in early flower development in *Antirrhinum* (Carpenter *et al*, 1995), the names of these *Antirrhinum* stages are shown in italics.

Stage	Landmark event at beginning of stage
Stage 1	Flower buttress arises *(Eye)*
Stage 2	Flower primordium forms *(Loaf)*
Stage 3	Sepal primordia arise *(Pentagon)*
Stage 4	Sepals overlie floral meristem *(Floritypic)*
Stage 5	Petal and stamen primordia arise *(Petal Mound)*
Stage 6	Sepals enclose bud
Stage 7	Long stamen primordia stalked at base
Stage 8	Locules appear in long stamens
Stage 9	Petal primordia stalked at base
Stage 10	Petals level with short stamens
Stage 11	Stigmatic papillae appear
Stage 12	Petals level with long stamens

The development of the floral meristem culminates in the production of a four whorled flower containing the perianth and reproductive organs. The perianth whorls contain the sepals (whorl 1) and petals (whorl 2). Reproductive organs arise in the centre of the floral meristem, and contain stamens in whorl 3 and carpels in whorl

four. The *Antirrhinum* flower is zygomorphic, and has bilateral symmetry. The flowers contain 5 sepals, 5 petals, only 4 stamens (since one extra stamen primordium is aborted early in development) and 2 fused carpels. In contrast *Arabidopsis* flowers are radially symmetrical and contain 4 sepals, 4 petals, 6 stamens and 2 fused carpels.

A complex network of genetic regulators control the transition from inflorescence to floral meristem and the subsequent formation and maturation of the floral organs. Many of these genes have been identified and the following section describes their roles.

1.3 The floral homeotic genes of *Arabidopsis* and *Antirrhinum*

1.3.1 Introduction

Bateson (1894) coined the term "homeosis" to describe the replacement of one member of a meristic series by another member of the series. A meristic series consists of a series of serially repeated related units and examples include the segments of insects or the whorls of flowers. Homeotic genes specify the identity of the different members of such series in both animals and plants. Thus in a homeotic mutant one member is transformed into another. For example in the *Drosophila* homeotic mutant *Antennapedia*, legs grow in the place of antennae. As might be expected for genes with such major developmental effects, homeotic genes encode proteins involved in regulating numerous target genes.

Two types of floral homeotic genes have been identified in flowering plants that have specific roles in floral development. The first group includes those floral homeotic genes that function to control the transition from inflorescence meristem to floral meristem. The second group of floral homeotic genes, the organ identity genes, are expressed in distinct domains within the flower during its development and specify organ identity within the concentric whorls of the flower. The genes controlling floral development have been highly conserved in evolution, as demonstrated by the

high degree of similarity between genes involved in floral development in *Antirrhinum* and *Arabidopsis.*

Genes with floral organ identity function are dominated by a large gene family all containing the MADS box DNA binding domain. This domain is a sequence of 56 amino acids which is highly conserved in many proteins but was first identified in the plant floral homeotic genes *AGAMOUS (AG)* and *DEFICIENS (DEF)* (Sommer *et al*, 1990; Yanofsky *et al*, 1990) the vertebrate *Serum Response Factor (SRF)* (Norman *et al*, 1988), and the yeast *MCM1* gene (Passmore *et al*, 1988). This functional domain was thus named the MADS box *(MCM1-AGAMOUS-DEFICIENS-SRF).* In floral homeotic genes the MADS domain is required for DNA binding to sequence motifs known as CArG boxes (Schwarz-Sommer *et al*, 1992; Huang *et al,* 1993),

1.3.2 Meristem identity genes

A set of early transcriptional regulators are involved in specifying the floral meristem. These floral meristem identity genes include *FLORICAULA (FLO)* (Carpenter and Coen, 1990; Coen *et al*, 1990) and *SQUAMOSA (SQUA)* (Huijser *et al*, 1992) from *Antirrhinum* and their respective *Arabidopsis* homologues *LEAFY (LFY)* (Weigel *et al*, 1992) and *APETALA 1 (AP1)* (Irish and Sussex, 1990). Floral meristem identity mutants are characterised by the replacement of flowers with inflorescence-like structures. For example *Antirrhinum flo* mutant plants can produce inflorescence meristems, however indeterminate shoots form in the axils of the bracts

instead of floral meristems. Each of these shoots can in turn produce further shoots in the axils of their bracts (Carpenter and Coen, 1990; Carpenter *et al*, 1990).

Expression of *FLO* and *LFY* is thought to mark the position of future floral meristems. *In situ* hybridisation experiments shows that *FLO* RNA is initially expressed in the bract primordia as early floral meristems are beginning to form, expression is then observed transiently in the sepal, petal and carpel primordia but not the stamen primordia. The *Arabidopsis* homologue of *FLO*, *LFY* is also expressed early, being present before the morphologically visible formation of a floral meristem (Weigel *et al*, 1992). By expressing *LFY* constitutively Weigel and Nilsson (1995) demonstrated that *LFY* is sufficient to determine floral fate.

The gene *SQUAMOSA (SQUA)* (Huijser *et al*, 1992) in *Antirrhinum* and its homologue *APETALA 1 (AP1)* (Irish and Sussex, 1990; Mandel *et al*, 1992; Mandel and Yanofsky, 1995) in *Arabidopsis* also have a role in determination of the floral meristem and are believed to act downstream of *FLO/LFY*. Both are members of the MADS box family of transcription factors. Wagner *et al* (1999) used steroid inducible activation of LFY+ activity to demonstrate that the early expression of *AP1* results from activation by *LFY*. In addition, activation of *AP1* after *LFY* steroid-induction was found to be independent of protein synthesis demonstrating that *AP1* is a direct downstream target of LFY

The *Arabidopsis* floral organ identity gene *AGAMOUS (AG)* also has an essential role in maintaining determinate growth within the floral meristem. *ag* mutant's flowers produce an indeterminate number of whorls, in contrast to wild-type flowers. To dissect the role of *AP1, LFY* and *AG* in the maintenance of floral determinacy Mizukami and Ma (1997) produced transgenic 35S-AG *lfy ap1* double mutants. Constitutive expression of *AG* in these plants was sufficient to promote a determinate floral fate independent of *AP1* or *LFY* (Mizukami and Ma, 1997). Furthermore *AG* must be present within the fourth whorl to direct determinacy. When *AG* is placed under *AP3* promoter control (pAP3::AG) in transgenic plants, misexpression occurs in the petals. In an *ag-3* background pAP3::AG plants express *AG* in whorls 2 and 3 but this is not sufficient to confer determinacy.

The analysis of meristem identity genes in *Arabidopsis* suggests that activity of *AG* (or *c* function activity, see section 1.3.3) increases towards the apex of the inflorescence (Irish and Sussex, 1990; Weigel *et al*, 1992). In addition a similar *c* function gradient has been observed in *fimbriata (fim) Antirrhinum* mutants (Ingram *et al*, 1997). One possibility is that a floral repressor function exists during vegetative development, which gradually declines to enable a floral meristem to form. The *EMBRYONIC FLOWER (EMF)* genes are candidates for such repressors (Chen *et al*, 1997). In *emf1* and *emf2* seedlings the promoter of *AG* is activated, suggesting that the wild-type role of these genes is the suppression of *AG*. It is possible that an early high level of *EMF1* and *2* initially represses flower-promoting genes like *LFY* and *AP1,* which are then unable to activate *AG*, the level of *EMF* then progressively

declines during development eventually allowing *AG* to be expressed and promote flowering.

1.3.3 The *abc* model for floral organ identity

Genetic and phenotypic analysis of floral homeotic mutations in *Antirrhinum* and *Arabidopsis* has led to a simple model, termed the *abc* model, that explains how floral organ identity genes control floral organ identities along the radial axis of the flower (Coen and Meyerowitz, 1991). The *abc* model of floral organ identity proposes that three sets of floral homeotic function exist that act combinatorially to specify the identity of the four whorls of the flower. Genes with *a* group homeotic function are expressed in the outer two whorls (the sepals and petals), they alone specify sepal development and in combination with *b* function genes specify petal development. Genes with homeotic *c* function specify stamen development along with the *b* group and alone specify carpel development. An additional postulate of the model, suggested by single and double organ identity mutant analysis, was that the *a* and *c* functions are mutually exclusive whereas the *b* function is independent of both (Bowman *et al*, 1991b; Coen and Meyerowitz, 1991; Meyerowitz *et al*, 1991). The flowers of triple mutants that lack *a, b* and *c* activity consist solely of cauline leaves, suggesting that leaves constitute the ground state (Bowman *et al*, 1991b). *SEPALLATA 1, 2* and *3* are an additional set of MADS box genes that have been found to be required for the *b* and *c* function (Honma and Goto, 2001).

Floral organ identity genes with *a, b* and *c* function have been characterised in *Arabidopsis.* Two genes, *APETALA1 (AP1)* and *APETALA2 (AP2),* have been identified which are required for the *a* function. *APETALA3 (AP3)* and *PISTILLATA (PI)* (Bowman *et al*, 1989; Bowman *et al*, 1991b) are required for the *b* function, and a single *c* function gene *AGAMOUS (AG)* (Yanofsky *et al*, 1990; Bowman *et al*, 1989) specifies the identity in the stamen and carpel whorls. The homologues of *Arabidopsis* floral organ identity genes with *b* and *c* function have also been identified in *Antirrhinum,* but the *a* function of *Antirrhinum* has diverged (section 1.3.6). The *Antirrhinum* homologues of *AP3* and *PI b* function genes are *DEFICIENS (DEF)* and *GLOBOSA (GLO)* (Schwarz-Sommer *et al*, 1990) respectively, whilst *PLENA (PLE)* (Schwarz-Sommer *et al*, 1990) is the *Antirrhinum c* function homologue of *AGAMOUS (AG).* All of the floral organ identity genes from *Arabidopsis* and their *Antirrhinum* homologues encode MADS domain DNA binding proteins, with the notable exception of *AP2.* The *Arabidopsis a* function gene *AP2* encodes a putative nuclear protein with two 66 amino acid repeat motifs which show similarity to a DNA binding domain found in ethylene response factors (Jofuku *et al*, 1994; Okamuro *et al*, 1997).

1.3.4 Control of floral organ identity genes in *Arabidopsis* and *Antirrhinum*: pattern initiation

The *Arabidopsis* meristem identity genes *LFY* and *AP1* are expressed in the early floral meristem (from stage 1 onwards) before floral organ identity gene expression is initiated at stage 3, indicating that they might activate organ identity genes in the floral meristem (Weigel *et al*, 1992; Mandel *et al*, 1992).

LFY and *AP1* have been shown to act synergistically to activate expression of *AG*, the *Arabidopsis c* function gene, in the centre of the floral meristem (Weigel and Meyerowitz, 1993). In the case of *LFY*, Busch *et al* (1999) have demonstrated that *AG* is a direct downstream target and that LFY has DNA binding activity. Two *LFY* binding sites were identified in the 2nd intron of *AG* and these were shown to be necessary for *LFY* directed *AG* expression *in vivo*. The meristem identity genes are also important for the initiation of *b* function expression in the floral meristem. Although in this case *LFY* is the major activator and *AP1* only has a minor role (Weigel and Meyerowitz, 1993). *LFY* is believed to act with another gene *UNUSUAL FLORAL ORGANS (UFO)* to activate the *b* function genes *AP3* and *PI*. *UFO* is expressed prior to organ identity expression and is required for the activation of both *AP3* and *PI* in the *b* domain (Ingram *et al*, 1995). However constitutive expression of *UFO* in *lfy* mutants is insufficient to restore petal and stamen identity, indicating that *UFO* can only function as a co-regulator with *LFY* (Lee *et al*, 1997). The role of *UFO* may be to provide regional specificity to the activation of *AP3* and *PI*. *LFY* is expressed throughout the floral meristem whereas *UFO* expression becomes localised to a ring believed to correspond to the *b* function domain (Ingram *et al*, 1995). In *Antirrhinum* an orthologue of *UFO* called *FIMBRIATA (FIM)* activates the *b* function genes *DEF* and *GLO* in whorls 2 and 3 (Ingram *et al*, 1997). *FIM* has been shown to encode an F-box protein also found in yeast cell cycle control factors. It is believed to activate and maintain the *b* domain by locally degrading *b* group repressors.

Another gene exists in *Arabidopsis* which controls the *b* domain, *SUPERMAN (SUP)* acts at the boundary of the third and fourth whorl to prevent proliferation of *AP3/PI* expressing cells (Sakai *et al*, 1995). Once initiated the *b* domain is believed to be maintained at least partly by auto-regulation (Jack *et al*, 1994).

Since *LFY* activation alone is insufficient to confer spatial restriction of the *a* and *c* function domains of the developing flower additional regulatory mechanisms must exist which ensure that these functions are separated.

An important premise of the *abc* model is that *a* and *c* function genes are antagonistic, producing mutually exclusive domains of action. This has been demonstrated in *Arabidopsis.* Loss of function mutants of the *Arabidopsis c* function gene *AG* are characterised by the conversion of stamens to petals and carpels to sepals suggesting expansion of the *a* function. In early (stage 1 and 2) floral meristems *AP1,* an *a* function gene is expressed in all four whorls, but expression becomes limited to the perianth whorls after stage 3 when *AG* begins to be expressed in whorls 3 and 4 suggesting that *AG* might repress *AP1* in these whorls (Mandel *et al*, 1992). Consistent with this hypothesis *AP1* is ectopically expressed in whorls 3 and 4 in *ag* mutants (Gustafson-Brown *et al*, 1994). Conversely ectopic *AG* is occasionally observed in the perianth whorls of strong *ap1* mutants suggesting that *AP1* may repress *AG* in the perianth whorls of wild-type plants (Weigel and Meyerowitz, 1993)

Ectopic expression of *AG* has also been observed in the perianth whorls of *ap2* mutants suggesting that the *AP2 a* function gene has a role in the repression of *AG* in these whorls (Drews *et al*, 1991). Ectopic *AG* expression is present in the first whorl of flowers from the strong *ap2* mutant *ap2-2,* from stage 2 (*i.e.* precociously) in flower development. In addition, variable and weak *AG* expression is observed ectopically in both whorl 1 and 2 of flowers from the weak *ap2* mutant *ap2-1* during later stages of flower development (Drews *et al*, 1991). Because *AP2* RNA is expressed in all four whorls of the flower throughout floral development its domain specific repression of *AG* must involve additional domain restricted factors.

LEUNIG (LUG) also represses early floral expression of *AG* in the perianth whorls (Liu and Meyerowitz, 1995). *lug* mutant flowers display weak homeotic transformation of petals and sepals towards stamens and carpels resulting from precocious and ectopic expression of *AG* throughout the floral meristem. In contrast to *AP2, LUG* does not have a role in the specification of sepals and petals since the *ag* phenotype is epistatic to *lug* with respect to floral organ identity. *LUG* collaborates with *AP2* to repress *AG* in the outer floral whorls since the partial ectopic expression of *AG* in the sepals of *lug* mutants is extended to 100% misexpression in *ap2-1 lug* double mutants.

Experiments using pAG::β glucuronidase (GUS) gene fusions showed that the *cis*-acting elements required for regulation of *AG* are intragenic (Sieburth and

Meyerowitz, 1997). Two constructs were produced, designated pAG::GUS and pAG-I::GUS, pAG::GUS contained 6 kb of upstream *AG* sequences but pAG-I::GUS also included 3.8kb of genomic sequence extending to exon 3. Whereas the pAG-I:GUS construct directed GUS expression in a pattern that mimicked that of the endogenous *AG* gene, the pAG::GUS construct did not.

The role of intragenic sequences in the regulation of *AG* had since been studied and the large third intron of *AG* identified as an important region for *cis*-acting regulation. Busch *et al* (1999) have shown that the 2nd intron alone is sufficient to direct the correct *AG* expression pattern. Bomblies *et al* (1999) have shown that *AP2,* a negative regulator of *AG,* interacts with several discrete sequences of the large 2nd intron of *AG.*

1.3.5 Maintenance of *abc* gene expression: the role of fate determination in floral development

Expression of the *a, b* and *c* organ identity genes is initiated early in floral development (stage 3) and persists until very late stages when organs are differentiating. For example the *Arabidopsis c* function gene *AG* is initially expressed strongly in the centre of the floral meristem at stage 3 (Drews *et al*, 1991) and is then uniformly expressed throughout the stamen and carpel primordia. Later in development, during morphological differentiation, *AG* expression becomes restricted to certain cell types within these whorls (Bowman *et al*, 1991a). In whorl three *AG* is becomes restricted to the connective tissue, walls and filaments of the anthers. In the carpel whorl *AG* is initially present in all cells of the ovule but later

becomes restricted to a single cell layer of the inner integument, termed the endothelium. The restriction of *AG* to specific particular tissues in mature stamens and carpels suggests a role in cell fate specification late in development. Studies of other organ identity genes have confirmed a late requirement for organ identity genes (Carpenter and Coen, 1990; Bowman, 1989). Zachgo *et al* (1995) used a temperature sensitive allele of the *Antirrhinum b* function floral homeotic gene *DEF* (*def*-101) to determine the temporal requirement of *DEF* during floral development and found that *DEF* was required both early and persistently during floral development for its role in cell fate specification thus shifts to a restrictive temperature late in floral development have effects on petal cell specification. Additionally in some cases late expression of floral homeotic genes is sufficient produce correct expression, independent from early expression (Carpenter and Coen, 1990). This suggests against models where the pattern of organ identity genes is initiated by transient early signals, and maintained only by auto-regulation as in this case *DEF* and *GLO* expression would not be activated. Therefore mechanisms must exist which maintain correct early patterns of floral homeotic genes, through many cell divisions, until the late stages of floral development. Two different types of mechanisms could conduct this process. Cell-signalling mechanisms could be involved which enables each cell to influence the identity of its neighbour so that patterns are maintained non-cell autonomously.

The majority of plants cells are believed to be reversibly fated and able to change fate in response to their new position (reviewed in Weigel and Doerner, 1996) and a

number of studies have revealed that during the earlier stages of floral development floral homeotic gene transcription factors act non-cell autonomously. Many of these reports have used genetic mosaics or chimeras in which the floral homeotic gene is only expressed in a certain clonal cell layer/or layers of the meristem. Cell-cell communication of a signal mediated by the *FLO* gene between layers of the meristem has been demonstrated (Hanke *et al,* 1995; Carpenter and Coen, 1995). *In situ* hybridisation using chimeras showed that *FLO* expression in one layer was sufficient to induce expression of the organ identity genes *DEF* and *PLE* in all three layers (Hanke *et al,* 1995). The homologue of *FLO, Arabidopsis LFY* can also act non-cell autonomously between and within layers to activate *AG* and *AP3* (Sessions *et al*, 2000). Non-autonomous action of *LFY* involved the movement of the protein between cell layers, presumably *via* plasmodesmata, channels which cytoplasmically connect plant cells. A mosaic study using the Cre/*loxp* site-specific recombinase system showed that *AG* can also act non-cell autonomously (Sieburth *et al*, 1998).

Therefore at least during the early stages of floral development the fate of cells is not determined but influenced by neighbouring cells. However a few studies provide evidence that during the course of floral development cells acquire increasing lineage restrictions that may reflect a more determined state. Using clonal analysis in *ap3-3* and *ag1* mutants Jenik and Irish (2000) distinguished two distinct phases in the control of cell division during floral development in *Arabidopsis.* In the first stage cell division was regulated in a manner dependent on the position of the cells within the FM. But after stage 6 division pattern became dependent on the identity of the

developing organs (Jenik and Irish, 2000). In another study, a temperature sensitive transposon was used to mark cells of the floral meristem at particular developmental times to study cell lineage restriction in *Antirrhinum* (Vincent *et al*, 1995). The results suggested that initial lineage restriction begins at the floritypic stage and later restrictions occur between the dorsal and ventral surfaces of the petals. Lineage restriction is not direct evidence of cell fate determination but could pave the way for irreversible cell commitment later on.

A few studies suggest that late in floral development cell fate becomes determined (Irish and Nelson, 1991; Bradley *et al*, 1996). The excision of an unstable transposon in *def*-621 results in patches of pigmented petal epidermal cells on transformed second whorl sepals (Carpenter and Coen, 1990). Tiny patches consisting of only four petal cells were observed and these were clonal. In other words cell fate appears independent of neighbouring cells.

The Pc-G genes of animals are involved in the maintenance of stable states of gene repression and include targets which regulate cell fate, such as homeotic selector genes. The action of Pc-G in locking into place states of gene expression may provide a mechanism for fate determination in which fate is independent of the environment. The identification of an *Arabidopsis* Pc-G gene called *CURLY LEAF* (*CLF),* which acts during the late stages of floral development to maintain repression of the organ identity gene, suggested that such a mechanism might be important during later stages of floral development (Goodrich *et al*, 1997, section 1.4.5.2).

1.3.6 Divergence of the *a* function between *Arabidopsis* and *Antirrhinum*

The homeotic *a* function has diverged between *Arabidopsis* and *Antirrhinum*. *Arabidopsis AP2* regulates organ identity in the outer floral whorls, but also has a major role in the repression of *c* function expression in these whorls. Recent studies (E.Keck, E.Coen and R. Carpenter) have shown that the *AP2*-like genes of *Antirrhinum* have little effect on *c* function expression. Two *Antirrhinum AP2* homologues have been identified and these have little effect on *c* function even as double mutants and in addition have very minor effects on cell fate. *Antirrhinum SQUA* is considered to be the homologue of the other *a* function gene in *Arabidopsis AP1*. *SQUA* has no identifiable effects on sepal or petal identity that can be separated from its role in the promotion of floral meristem identity. In *squa* mutants, flowers are occasionally produced, but these have normal organ identity in whorls 1 and 2.

Arabidopsis a function genes also fail to conform strictly to the *abc* model. Neither strong nor weak *Arabidopsis a* mutants produce a strict carpel, stamen, stamen, carpel pattern. Weak mutants show partial homeotic transformations whereas the second whorl is often absent in strong alleles. In summary, analysis of the *Antirrhinum* and *Arabidopsis a* function therefore suggests that this function is phylogenically less well conserved than *b* or *c* functions.

The *a* function may predominantly be required to establish a floral meristem. The perianth organs of plants are a recent addition to the plant kingdom and have evolved

independently in a number of plant species, in contrast stamens and carpels are evolutionarily ancient and have homologues in non-flowering plants. Examination of MADS box gene phylogeny in a number of species suggests that *b* and *c* functions had ancient roles in reproduction (Theissen *et al*, 2000). The *a* function gene *AP2* is not a MADS box gene but again its original role may have been reproductive. The expression of *AP2, Antirrhinum AP2LIKE1* and petunia *AP2* homologues in ovules suggests an involvement in seed development (Theissen *et al*, 2000). In addition *ap2*-mutants have a seed coat phenotype (Jofuku *et al*, 1994).

1.3.7 Comparison of the *c* functions of *Arabidopsis* and *Antirrhinum*

PLENA (PLE) is the functional *Antirrhinum* homologue of the *c* function *Arabidopsis* gene *AG* (Bradley *et al*, 1993). A transposon tagged *ple* allele was obtained in a transposon mutation experiment (Carpenter and Coen, 1990), and shown to be allelic to the classical *plena* mutation (Stubbe, 1966). *ag* and *ple* mutant flowers are similar, both being characterised by reiterating whorls of sepals and petals. A pattern which presumably results from the expansion of the *a* function into all four whorls together with loss of floral determinacy.

The expression of *PLE* during floral development mirrors that observed for *AG*. Expression begins in the centre of the floral meristem at the florotypic stage when sepal primordia become visible. Expression then persists in the stamen and carpel whorls until the late stages of organ differentiation. At this time *PLE* is present within specific areas of the stamen whorl such as the stamen filaments, connective

tissue and anther wall. In the fourth whorl *PLE* becomes restricted to the ovules and at later stages confined to the integuments.

Identification of *ovulata*, a mutant corresponding to a gain of function allele of *PLE* (Bradley *et al*, 1993) provided an opportunity to examine the effects of ectopic *PLE* expression. In *ovulata* mutants *PLE* is expressed in the inflorescence apex and the early floral meristem. Expression intensifies at the florotypic stage in both the dome of the floral meristem and whorl 1. Later, expression occurs in all four whorls of the developing flower. Ectopic *PLE* in the outer whorls transforms sepals into carpels and petals into stamens. Vegetative *PLE* expression in *ovulata* occurs in the leaves, stems and roots. Ectopic expression of *PLE* within vegetative organs does not lead to the widespread curling of leaves observed when *AG* is expressed constitutively in *Arabidopsis*. Instead bracts are sometimes deformed and very rarely stigma like cells are observed at leaf tips.

The opposing phenotypes of *plena* and *ovulata* were shown to result entirely from the opposite orientation of the Tam3 transposon insertion within the large second intron of *PLE* (Bradley *et al*, 1993) in *ple* loss of function and *ovulata* alleles. Tam3 contains a transcriptional terminator which operates only when Tam3 is in the same orientation as the host gene, thus explaining the loss of function *plena* mutation. When in the opposite orientation read through of Tam3 occurs along with normal splicing of the *PLE* transcript to produce the gain of function *ovulata* mutant.

To explain ectopic expression of *PLE* in *ovulata* mutants Bradley *et al* (1993) proposed that negative regulation in *PLE* was intragenic, so that the action of a negative regulator element of *PLE* is disrupted by the insertion of the Tam3 element. Tam3 insertions more than 400bp apart can produce *ovulata* phenotypes however, so that multiple or complex regulatory elements may occur. The regulation of *Arabidopsis AG* has since also been found to require corresponding intragenic regions.

A second *c* function gene has been identified in *Antirrhinum* called *FARINELLI (FAR).* A mutant of *far* has been identified in a reverse genetic screen (Davies *et al*, 1999). The primary sequence of *FAR* shows extensive similarity with the *Arabidopsis c* function floral organ identity gene *AG* and is more similar to *AG* than *PLE* is to *AG.*

Flowers of *far* single mutants do not have a phenotype similar to the *c* function mutants *ag* or *ple*, being similar to wild-type, but show a partial loss of male fertility. The *far ple* double mutant proved more informative, the fourth whorl of these flowers has five normal petals as opposed to the *ple* single mutant flower, which shows variable fourth whorl transformation but often has two or three sepaloid/petaloid/carpeloid organs. Further analysis revealed that *FAR* and *PLE* act in a partially redundant manner to exclude *b* function genes from the fourth whorl. *FAR* is not required for inner whorl specification like *PLE* however loss of *FAR* does

enhance petaloidy of third whorl organs present in weak *ple* mutants suggesting a minor role in stamen identity.

The phenotype of *Arabidopsis* and *Antirrhinum c* function mutants differs. In the *Arabidopsis ag* mutant a new flower is initiated after 3 whorls. In contrast *ple* mutants produced four whorls before a new flower is initiated (Bradley *et al*, 1993; Bowman *et al*, 1989). In addition, *Antirrhinum* loss of *b* function mutants lack the fourth floral whorl, a feature not seen when *b* function is lost in *Arabidopsis* (Trobner *et al*, 1992). Davies *et al* (1999) suggest that these differences result from two unique features of *b* and *c* control in *Antirrhinum* (Davies *et al*, 1999). The first suggestion is that *OCTANDRA (OCT),* a candidate for *SUP* function in *Antirrhinum,* is dependent on *c* function to exclude *b* function genes from the fourth whorl. *SUP* in contrast is independent of *c* function. The *c* function dependency in *Antirrhinum* involves both *PLE* and/or *FAR.* In *Arabidopsis* loss of *AG* and continued regulation of the *b* domain results in absence of *b* or *c* function from the fourth whorl, thus a new flower initiates in its place. In *Antirrhinum b* function expands slightly to the fourth whorl due to a reduction in *PLE* and *OCT* activity. However *FAR* still remains enabling partial production of a fourth whorl before a new flower forms.

1.3.8 Mutations that confer altered *c* function expression in *Antirrhinum*

A number of genes have been identified that repress *PLE* in the perianth whorls. The pleiotropic effects of mutation of these genes results from their *PLE* dependent and independent functions.

Flowers of the recessive mutant *fistulata (fis)* have reduced and stamenoid petals (McSteen *et al*, 1998). *In situ* hybridisation experiments indicate that staminoidy of the petals is correlated with ectopic *PLE* expression in whorl two. *FIS* shares a partially redundant role in the negative regulation of perianth *PLE* expression with another gene *HEPTANDRA (HEP).* Stamenoidy occurs in the petals of *hep* plants and an additive phenotype exists in *fis hep* double mutants (McSteen, 1997).

Another gene with a potential role in *PLE* regulation is *POLYPETALA (POLY).* Only one mutant allele is known, *poly-*, and this is semidominant (McSteen, 1997; McSteen *et al*, 1998). *poly-/poly-* homozygotes have flowers with a normal outer sepal whorl, but internal to this is an indeterminate number of petals. *poly-/Poly+* heterozygote plants have flowers with an intermediate phenotype, usually with 5 whorls of organs in the order, sepal, petal, petal, stamen (often with petaloid tissue), carpel. *In situ* analysis shows that *PLE* expression is downregulated in mutants which might account for the indeterminacy and increased petalloidy in internal whorls. However *POLY* has an additional role in the formation of organ primordia that is not dependant on its role in regulation of *PLE* because plants homozygous for *poly* have

additional defects in the formation of organ primordia that are not present in *ple* mutants (McSteen, 1998). If the semi-dominant *poly-* allele represents a gain of function mutation, then the wild-type function may include repression of *PLE* in whorls 1 and 2. In dominant gain of function mutations, this activity would extend to whorls 3 and 4, resulting in *ple-* aspects of the phenotype.

1.3.9 Dorso-ventral patterning and the regulation of *PLE*

Arabidopsis flowers are radially symmetrical whereas *Antirrhinum* flowers are zygomorphic. Zygomorphy describes the presence of asymmetry dependant on a dorso-ventral axis. The flowers of *Antirrhinum* have three types of petals, two upper dorsal petals, a lateral petal on each side and one lower ventral petal. Dorsoventrality also affects the stamen whorl causing the abortion of the dorsal stamen early in its development.

Three genes have been isolated that control this dorsoventral pattern *CYCLOIDEA (CYC), DICHOTOMA (DICH)* and *DIVARICATA (DIV)* (Luo *et al*, 1996; Almeida *et al*, 1997; Luo *et al*, 1999). *cyc dich* double mutants produce radially symmetrical flowers which have 6 ventral petals. Single *cyc* mutants produce an intermediate semi-peloric phenotype. Loss of *dich* only affects the dorsal petals indicating that *CYC* has a more dominant role. Both *CYC* and *DICH* are expressed in a dorsal domain, although *DICH* expression is more limited. *DIV* is required for ventral identity; it is negatively regulated by *CYC* and *DICH.*

Genes that control dorso-ventral patterning in *Antirrhinum* also regulate floral organ identity genes. Mutants of the *PLE* regulator *FIS* displays altered morphology and floral identity in a dorso-ventral pattern. The ectopic *PLE* expression seen in *fis* mutants begins at a ventral location on the petal primordia at stage 5 and later extends along the length of the adaxial surface of ventral and lateral petals, in a one to two layer strip. Dorsal specific inhibition of *PLE* may involve *CYC* or *DICH* genes as both are expressed dorsally and required for dorso-ventrality

The semidominant *cyc* mutant allele *backpetals* results from a transposon insertion in the promoter region of *CYC* (Luo *et al*, 1999). In *backpetals* lateral and ventral petals are dorsalised. This results from the late ectopic expression of *CYC* in both lateral and ventral petals. It is plausible that transposon insertion disrupts a *cis* regulatory region usually required for the repression of *CYC*. Since ectopic expression only occurs late, this repressor might normally only be required for late maintenance, a feature of Polycomb-group genes.

1.4 Polycomb group genes in plants and animals

1.4.1 Introduction

CLF was the first Polycomb-Group gene to be identified in plants and is a homologue of the *Drosophila* gene *Enhancer of Zeste (E(z)).* The product of *E(z)* is a Polycomb-group protein, a transcription factor responsible for the correct pattern of silencing of homeotic genes and other targets. An additional *E(z)* homologue has been identified in *Arabidopsis thaliana* termed *MEDEA (MEA)* and is one member of the *FERTILISATION INDEPENDENT SEED (FIS)* class of genes which regulate cell proliferation during seed development.

Recent evidence has emerged which suggests that the interaction of E(z) with another Pc-G protein Extra sex combs (Esc) is conserved in plants and animals. In addition the E(z)/Esc complex is distinct from other Pc-G complexes and may have an essential early role, initiating Pc-G silencing after early acting gap gene repressors.

The mechanism of Pc-G silencing is a subject of much debate. It is believed that Pc-G proteins complexes modify higher-order chromatin structure to silence their target genes. The heritable nature of this silencing may involve a heritable "tag", a specific modification on the chromatin of silenced targets which enables the silenced state to be faithfully propagated to all new daughter cells.

1.4.2 The Polycomb-group of genes

Polycomb-group (Pc-G) genes are a large group of genes first identified in *Drosophila melanogaster* on the basis of a common genetic function (reviewed in Pirrotta, 1997; Pirrotta, 1998; Paro, 1995; Hagstrom and Schedl, 1997). Genes of the Pc-G group were originally studied as weak loss of function mutations that produced adult homeotic transformations such as ectopic sex combs on the legs. An example is *Extra sex combs (Esc)* in which the second and third thoracic legs are transformed towards the pattern of the first thoracic leg resulting in the production of extra sex combs in males (Struhl, 1981). These genes act to maintain the correct boundaries of gene repression of the homeotic genes and other developmental targets during animal development. Since Pc-G loci are expressed both maternally and zygotically both maternal and zygotic products must be removed to reveal their roles during embryogenesis. Loss of function mutations in *Drosophila* Pc-G genes cause ectopic expression of homeotic products along the anterior-posterior axis, for example a loss of function mutation in the Pc-G gene *Polycomb (Pc)* disrupts anterior-posterior patterning such that all thoracic and abdominal segments are homeotically transformed into eighth abdominal segments (Simon *et al*, 1992).

The identity of the body segments of *Drosophila* depends on the precise combination of the Antennapedia (ANT-C) and Bithorax (BX-C) homeotic genes that are active. The correct pattern of ANT-C and BX-C transcription is initiated early by gap and segmentation transcription factors, which divide the embryo up into fine stripes which define boundaries of homeotic gene expression. An example of such a gap

gene is *hunchback* (*hb*), which represses transcription of the homeotic gene *Ultrabithorax (Ubx)* in the anterior portion of the embryo (Zhang and Bienz, 1992). In common with other segmentation gene products expression of *hb* has disappeared by mid-germ band elongation. Because the activities of the ANT-C and BX-C genes are required throughout development in order to specify the fate of each cell under their control an additional set of genes are required to maintain these early patterns. The two groups responsible are the Polycomb (Pc-G) and trithorax-group (trx-G) of genes. The Pc-G maintain the correct pattern of homeotic repression whereas the trithorax-group (trx-G) are antagonistic to the Pc-G group and retain the correct profile of homeotic activation. Thus Pc-G mutations do not affect the initial expression patterns of their targets, however maintenance of the pattern breaks down about 5 hours into embryogenesis.

1.4.3 Polycomb-group mediated repression

1.4.3.1 Introduction

The protein products of Pc-G genes are believed to act together in multiprotein complexes to heritably repress their targets by regulating higher-order chromatin structure. Several key questions remain to be answered before their mechanism of action can be understood. In particular: 1) what is the signal required for formation of an initial Pc-G repression complex; 2) what is the nature of this complex and how does it alter chromatin structure in order to silence genes; and 3) how is repression "remembered" retained through DNA replication and cell division, *i.e.* what is the heritable "tag" that persists through mitosis?

1.4.3.2 Initiation of Pc-G mediated silencing

Most Pc-G proteins have been found to lack specific DNA binding activity when tested in *in vitro* assays and are believed instead to bind to the chromatin of specific *cis*-regulatory regions called polycomb response elements (PREs) most likely through protein-protein interaction. PRE elements have been identified in the regulatory regions of many Pc-G target genes in *Drosophila.* When these elements are placed upstream of a reporter construct, for example the mini-white gene, they confer Pc-G dependant repression to the transgene (Simon *et al*, 1993). In addition immunolocalisation shows that the introduction of a PRE containing transgene to a novel genomic location leads to Pc-G protein binding at this site (Chan *et al*, 1994). Once bound to PREs a single Pc-G protein may recruit additional Pc-G proteins to the PRE, by means of protein-protein interaction, to form a functional Pc-G complex. Muller *et al* (1995) demonstrated that Polycomb (Pc) fused to the GAL4 DNA-binding domain can silence a reporter gene containing GAL4 binding sites, presumably because Pc-GAL4 is tethered to the reporter. The silencing was known to require additional Pc-G proteins, suggesting that Pc had recruited Pc-G proteins to form a complex. This mechanism may explain the nucleation of a Pc-G complex but does not reveal how Pc-G genes initially become bound to the PREs of their specific target genes. The identification of a Pc-G protein with sequence specific DNA binding activity may provide an answer. The *Drosophila* Pleiohomeotic (Pho) Pc-G protein shares significant homology to YY-1 a mammalian transcription factor and has been shown to bind DNA (Brown *et al*, 1998). Fritch *et al* (1999) show that Pho binds to sites in the *Ubx* PRE and that mutation of these sites abolishes Pc-G

repression *in vivo*. However, Poux *et al* (2001) argue that Pho does not directly recruit other Pc-G proteins to the PRE but instead may alter the chromatin structure to facilitate interactions.

The formation of a Pc-G complex at a PRE is believed to be a highly cooperative process. The extent of silencing exerted by a PRE on a transgene differs depending on its site of insertion (Reviewed in Hagstrom *et al*, 1997; Fauvarque and Dura, 1993), indicating perhaps that cooperation with adjacent PREs strengthens silencing. Transgenes containing PRE's inserted at ectopic positions can exert long range silencing effects (Zink and Paro, 1995; Chan *et al*, 1994). We might imagine a Pc-G complex forming at a strong PRE and then searching the DNA for weaker PREs in order to increase its sphere of influence. *Trans* interactions have been implicated by experiments using PRE containing transposon constructs to transform *Drosophila*. Flies homozygous for these elements produced stronger and more stable PcG complexes (Fauvarque and Dura, 1993; Kassis, 1994; Chan *et al*, 1994) indicating an interaction between the two paired homologues.

Since Pc-G proteins "take over" from the gap protein Hunchback (Hb) at a number of homeotic regulatory elements it might follow that Hb provides a recognition and recruiting element for the formation of a Pc-G complex (Busteria and Bienz, 1993). An absolute requirement of Hb in this establishment is unlikely since PRE constructs lacking Hb binding sites can establish silencing (Zink and Paro, 1995). Additional evidence that Pc-G silencing can be established independent of Hb was gained from

examination of silencing on the regulatory region of the *Drosophila Ultrabithorax (Ubx)* gene, a homeotic gene that is regulated by Pc-G proteins *in vivo*.

Poux *et al* (1996) made transgenes that combined early and late-acting enhancers of the *Drosophila* homeotic gene *Ubx* with a strong PRE. The late enhancer PRE construct combination caused repression of a reporter gene in all parts of the embryo, even in tissues where *hb* is not expressed. When both early and late enhancers were combined with the PRE, repression is established only in cells where the reporter gene is inactive at early stages *i.e.* regions where the early enhancer is inactive (Poux *et al*, 1996). These results demonstrated that the activity state of the gene at the time of Pc-G complex formation heavy influenced the pattern of silencing. In addition these results showed that Hb was not required for initiation of Pc-G silencing on the *Ubx* gene. However it is possible that Hb can contribute to Pc-G silencing initiation based on its interaction with Mi-2 (see below).

There is further evidence, which suggests that the transcription status of a target gene determines whether a Pc-G repression complex can become established. A number of studies have shown that a repressive Pc-G complex cannot become established on a transcriptionally active site (Poux *et al*, 1996; Zink and Paro, 1995; Cavalli and Paro, 1998; Poux *et al*, 2001). Silencing by a PRE element placed adjacent to a gene inducible by GAL4 can be overcome by increasing the amounts of GAL4 (Zink and Paro, 1995). In addition, a short pulse of activation during embryogenesis can release PcG dependent silencing. The resulting activation is

mitotically inherited and in some cases can be transmitted through meiosis (Cavalli and Paro, 1998). These results clearly show that once Pc-G repression is disrupted it is actively prevented from re-initiating silencing. Activation continues long after GAL4 has decayed indicating that other factors are involved in this maintenance. trx-G proteins, the antagonists of PcG proteins present the ideal candidates for this role.

trx-G proteins are the antagonists of Pc-G silencing but surprisingly have been found to act in close proximity to Pc-G complexes on PREs. Multiple binding sites for the *Drosophila* trx-G protein GAGA have been found on a number of PREs, and *in vivo* GAGA strongly co-localises with Pc on PREs (Strutt *et al*, 1997). In addition cross-linked chromatin analysis has revealed co-localisation of the trx-G protein Trithorax (Trx) and Pc-G proteins *in vivo* (Orlando *et al*, 1998). Because trx-G and Pc-G proteins co-localise extensively on chromosomal elements direct competition as a mechanism of function is unlikely,

In summary, initiation of Pc-G mediated silencing is not well understood, Pc-G complexes may be recruited to specific targets though interaction with Pc-G members with DNA binding specificity (e.g. Pho) or perhaps other transcription factors such as Hb. Transcriptional activation may generate chromatin structures that are inaccessible or resistant to Pc-G proteins.

1.4.3.3 Two biochemically distinct Pc-G complexes occur

Biochemical analysis suggests that Pc-G proteins are present in two distinct complexes. Each of these complexes has been identified both in *Drosophila* and

mammals (Jones *et al*, 1998; van Lohuizen *et al*, 1998; Sewalt *et al*, 1998). The two complexes appear to have different functions and may act at different times in development.

One of the complexes contains the products of the Pc-G genes *Enhancer of zeste (E(z))* and *Extra sex combs (Esc).* This complex is believed to be required at the initiation stage of Pc-G complex formation. The evidence for this is partly based on the transient early essential requirement for the *Esc* gene. *Esc* is unique amongst *Drosophila* Pc-G genes since it is only required and expressed during early embryogenesis (Simon *et al*, 1995). It is also unique because it only has ANT-C and BX-C genes as its targets *i.e.* it is more specific than other Pc-G members. *Esc* is crucial for Pc-G repression during a short period of embryogenesis, however the absence of *Esc* before or after this critical period does not produce any phenotypic defects (Struhl and Brower, 1982; Simon *et al*, 1995). Mutations in the mouse *Esc* homologue *embryonic ectoderm development (eed)* have a mid-gastrulation lethal phenotype also suggesting an essential early role (Schumacher 1996). It is possible that Esc is required to provide an essential bridging role between gap-gene mediated repression and the formation of a Pc-G complex, so that it is no longer needed once Pc-G proteins are recruited. Although *E(z)* forms part of the initiating Pc-G E(z)/Esc complex it does not simple have an early role like *Esc*. *E(z)* has been shown to be required continuously, like other Pc-G members, in order to maintain the repression of homeotic gene targets (Jones and Gelbert, 1990; Phillips and Shearn, 1990; Shearn *et al*, 1978). In addition *E(z)* is continuously required to maintain the chromosome

binding of other Pc-G proteins, including component Pc-G proteins of the Pc-G maintenance complex PRC1 (Rastelli *et al*, 1993; Platero *et al*, 1995).

A Pc-G complex named PRC1 is believed to be required to maintain Pc-G silencing at targets where Pc-G silencing has been initiated. In *Drosophila* this Pc-G complex contains the Pc-G proteins Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and Sex combs on midlegs (Scm) (Shao *et al*, 1999) and in humans a similar complex exists which contains human polyhomeotic (HPH), human polycomb 2 (HPC2), BMI1 and RING1 (Van Lohuizen *et al*, 1998; Sewalt *et al*, 1998).

The two different Pc-G complexes also appear to be functionally distinct. Van der Vlag and Otte (1999) have demonstrated that the histone deacetylases HDAC1 and HDAC2 associate and are necessary for the function of the E(z)/Esc complex but that these histone deacetylases do not interact with Pc-G proteins of the PRC1 complex.

1.4.3.4 The role of chromatin remodelling in Pc-G function

Chromatin structure has an important role in gene regulation. Changes in chromatin structure can induce a more "open" conformation, believed to be more accessible to gene activation or a more repressive conformation, favouring the repression of targets. Chromatin structure can be altered in a number of ways, including the post-translational modification of histones (the component proteins of chromatin which form octamer complexes called nucleosomes), DNA methylation and as a result of chromatin remodelling complexes. The chromatin modification that facilitates the action of both Pc-G complexes and antagonist trx-G complexes is believed to involve

the recruitment of ATP-dependant chromatin-remodelling complexes (reviewed in Guscin and Wolffe, 1999; Aalfs and Kingston, 2000). It has become clear that chromatin-remodelling complexes are not merely chromatin 'opening' complexes but gene specific remodellers that can also have repressive roles. The ATP dependent chromatin-remodelling complex SWI/SNF was initially discovered in the yeast *S.cerevisae* but related complexes have been discovered in both *Drosophila* and mammals indicating evolutionary conservation.

There is increasing evidence that chromatin-remodelling complexes are involved in trx-G regulation in *Drosophila*. Firstly, the *Drosophila* trx-G proteins *Brahma (Brm), Osa* and *Moira* have been identified as subunits of the *Drosophila* Brm complex, which is similar to the SWI/SNF chromatin remodelling complex of yeast (Tamkun *et al*, 1992). Secondly, the *Drosophila* SET domain trx-G protein Trx and its human homologue ALL1 both interact *in vitro* with homologues of a yeast SWI/SNF protein subunit called snf5 (Rosenblatt-Rosen *et al*, 1998). In addition, using chromatin remodelling assays Shao *et al* (1999) detected competition between a human SWI/SNF complex and the Pc-G complex PRC1 for a nucleosome template. Thus trx-G complexes may antagonise Pc-G silencing by recruiting chromatin remodelling complexes or influencing these complexes to favour production of a more 'open' chromatin conformation. Pc-G complexes may also interact with chromatin remodelling complexes. Initial evidence for this is that the mammalian E(z) homologue EZH2 physically interacts with a protein with structural similarity to the DNA helicase present in the SWI/SNF complex (Cardosa *et al*, 1998).

Deacetylation of histones, the subunits that make up chromatin, has a role in Pc-G silencing. The acetylation of lysine residues on the amino terminal tails of histone neutralises their positive charge and may reduce their affinity for negatively charged DNA. This open chromatin conformation facilitates active gene expression, whereas deacetylation favours the repression of transcription. The enzymes that alter the acetylation state of chromatin are named acetyl transferases (HATS) and histone deacetylases (HDACs) respectively. Both exist in large multiprotein complexes which contain DNA binding proteins to direct them to specific genes.

Components of these complexes are found in mammals, Drosophila, *C.elegans* and plants. The human deacetylase complex NuRD also has ATP dependant nucleosome remodelling activity which is conferred by a subunit called Mi-2. In *Drosophila* the interaction of Hb with dMi-2, the *Drosophila* homologue of Mi-2, has a functional role in PcG silencing *in vivo* (Kehle *et al*, 1998). The *Arabidopsis* homologue of *Drosophila dMi-2* is *GYMNOS*, a gene that acts redundantly with *CRABS CLAW* to establish polarity in the carpel (Eshad *et al*, 1999).

Recruitment of a histone deacetylation complex is required for the function of Eed, the mouse homologue of Esc, which interacts both *in vitro* and *in vivo* with HDAC's (van der Vlag and Otte, 1999). Histone deacetylase activity co-immunoprecipitates with the Eed protein. The functional significance of deacetylation was confirmed by demonstrating that the deacetylase inhibitor Trichostatin A relieves Eed specific

repression. HDAC proteins fail to interact with other vertebrate Pc-G proteins reinforcing the belief that Esc, as part of the Pc-G complex with E(z), has a distinct initiation function. This may involve early deacetylation to provide an inactive gene template for Pc-G formation.

A histone deacetylase has recently been confirmed as a subunit of the *Drosophila* E(z)/Esc complex. The recent report identified two important subunits of the 600 kDa E(z)/Esc *Drosophila* complex (Tie *et al*, 2001). Purification of the E(z)/Esc complex revealed association with the histone deacetylase RPD3 and the histone binding protein p55. p55 has also been identified as a component of chromatin remodelling complexes and can bind directly to histone H4. E(z) and RDP3 are bound to a PRE of the *Ubx* gene *in vivo*, where RDP3 is required for correct PRE silencing. The authors suggest that p55 recruits a deacetylation complex to the E(z)/Esc complex to direct deacetylation of specific histone subunits.

In summary, the E(z)/Esc complex may act by recruiting histone deacetylases which leads to the production of a repressive chromatin structure. The antagonist trx-G group of genes, in contrast, may recruit chromatin-remodelling complexes to produce a more "open" chromatin structure favouring the activation of targets.

1.4.3.5 The heritable nature of Pc-G silencing

How do Pc-G proteins overcome the hurdles of DNA replication and mitosis? The clonally heritable nature of this silencing requires both self-propagation during DNA replication and a way of marking silenced PREs through the rest of the cell cycle. In

common with many transcription factors Pc-G proteins are likely to dissociate from the condensed chromosomes as cells move into mitosis. This dissociation has been demonstrated for the PcG proteins Pc, Ph and Psc which dissociate from chromosomes during mitosis, based on immunolocalisation (Buchenau *et al*, 1998)

Two alternatives exist; firstly that Pc-G silencing alters chromatin structure in a self-renewing manner which no longer requires the Pc-G complex for silencing maintenance. This is unlikely since silencing is lost if a PRE is excised using a FLP recombinase (Busteria *et al*, 1997). As a second option, a nucleus of proteins or a recognisable chromatin modification may remain during DNA replication and cell division to "tag" the target gene for reassembly of a Pc-G complex. Both acetylation and methylation of histones would provide ideal choices for the latter form of recognition tag. The semi-conservative nature of DNA and nucleosome dispersal during DNA replication could provide the initial memory but this would also need to be recognised in daughter cells so that the newly synthesised DNA strand or nucleosomes could be modified in the same way. In the case of DNA methylation, maintenance methylases recognise hemi-methylated DNA (as results from the replication of symmetrically methylated CpG sites) and restores symmetrical methylation on both DNA strands.

Methylation of specific histones has now been identified as a mechanism which could provide a heritable 'tag' to chromatin targets of vertebrate homologues of the *Drosophila* gene *Supressor of variegation 3-9* (*Su(var) 3-9*). In *Drosophila* this gene

is a modifier of an epigenetic phenomena called position effect variegation involving the variable spread of heterochromatin (compacted, late replicating chromatin) to juxtaposing euchchromatic regions. The mammalian homologues of *Su(var)3-9*, human *SUV39H1* and murine *Suv39h1* selectively methylate lysine 9 of the amino terminus of histone H3 *in vitro,* an activity conferred by an active methyltransferase mapped to the conserved SET domain and adjacent cysteine regions (Rea *et al*, 2000). The result is significant since covalent modifications of histone tails including acetylation, deacetylation, phosphorylation and methylation have been suggested to confer particular higher-order chromatin states which may be linked to epigenetic phenomena. In addition this specific histone methylation has recently been shown to create a binding site for the HP1 protein (Lachner *et al*, 2001; Bannister *et al*, 2001). HP1 is a chromo-domain protein implicated in heterochromatic gene silencing and had previously been found to interact with the *SUVAR* genes (reviewed in Jones *et al*, 2000). The association of HP1 with 'tagged' histones suggests a mechanism for the heritable propagation of silenced heterochromatin since HP1 can associate with SUV39H1. The HP1 bound to methylated histones will also be associated with SUV39H1 which will methylate newly deposited histones. These in turn will become bound by HP1, propagating the silenced state (Bannister *et al*, 2001). Although the PEV genes like *Su(var)3-9* are not strictly Pc-G genes (which in contrast mainly localise to the euchromatin) they do share common structural motifs with Pc-G proteins (the chromodomain and the SET domain) and may have functional similarities underlying their epigenetic effects on gene silencing.

1.4.4 *E(z)* is an ancient gene with diverse functions

E(z) has diverse and global regulatory roles in *Drosophila*. It was initially classified a Pc-G protein in *Drosophila* as a result of mutant studies which revealed ectopic expression of homeotic genes and anterior to posterior segment phenotypic transformations (Jones and Gelbert 1990, Simon *et al* 1992). La Jeunesse and Shearn argue that E(z) can act both as a Pc-G protein and trx-G protein depending on the developmental context (La Jeunesse and Shearn, 1996). Recently mutations in a subset of Pc-G genes have been found to enhance trx-G phenotypes (Gildea *et al*, 2000). This subset includes E(z) providing further evidence that E(z) may have activation as well as repression roles. It is also of note that the SET domain, a conserved domain of E(z) is also present in genes of antagonistic function *i.e.* Pc-G genes and trx-G genes including *Trx*.

The ~130 amino acid SET domain present at the carboxy terminus of E(z) and its homologues was named after the three founding members Su-Var-3-9 (Tschierch *et al*, 1994), E(z) (Jones and Gelbert 1993), and Trx (Chinwalla *et al*, 1995). The high evolutionary conservation of this domain indicates that it is ancient and functionally significant. The SET domain is found in proteins involved in a variety of epigenetic mechanisms. For example the SET protein CLR4 of *S. pombe* is a modifier of centromeric position effects (Ivanova *et al*, 1998) and MES 2 in *C.elegans* regulates transgene silencing in the germline (reviewed in Seydoux and Strome, 1999) .

Pc-G silencing by E(z) has been linked to position effect variegation (PEV). The E(z) SET domain is also found in Su(var)3-9 a dominant suppressor of PEV in *Drosophila* (Tschiersch *et al* 1994). Extra copies of E(z) or its human homologue EZH2 can enhance PEV in transgenic flies (Laible *et al*, 1997). In the same study, expression of either human EZH2 or murine Ezh1 was demonstrated to restore telomeric silencing in *S.cerevisiae set1* mutants. These SET domain proteins probably substituted for the activity of SET1, a yeast SET domain protein implicated in telomeric silencing (Nislow *et al*, 1997). These results suggest conservation of silencing mechanisms between eukaryotes and additionally demonstrate the ancient and conserved role of the SET domain.

1.4.5 Polycomb genes in plants

Introduction

Two *E(z)* homologues have been identified and characterised in the plant species *Arabidopsis thaliana*, these are *CURLY LEAF (CLF)* and *MEDEA (MEA)* (Goodrich *et al*, 1997; Grossniklaus *et al*, 1998). Homologues of *E(z)* have also been discovered in humans, mice and *C. elegans* (Laible *et al*, 1997; Holdeman *et al*, 1998). An *Arabidopsis* homologue of the *Drosophila Esc* Pc-G gene called *FERTILISATION INDEPENDENT ENDOSPERM (FIE)* has also been identified and characterised. FIE and MEA interact demonstrating that the E(z)/Esc Pc-G complex has been conserved between plants and animals.

1.4.5.1 CURLY LEAF (CLF) maintains AG expression in Arabidopsis

The recessive *curly leaf-2 (clf-2)* mutant was obtained from a transposon mutagenesis experiment (Goodrich *et al*, 1997) and confers pleiotropic effects in vegetative and floral tissue. The vegetative phenotype of *clf-2* consists of narrow leaves which curl upwards along the longitudinal axis. Whereas *clf-2* flowers display partial homeotic transformations of the sepals and petals towards carpels and stamens respectively. The similarity of this phenotype to that observed when *AG* was constitutively expressed (Mizukami and Ma, 1992) lead to expression studies, which revealed ectopic *AG* expression in *clf* mutants. *AG* was ectopically expressed throughout the leaves and also present at a low level in the vasculature of the cotyledons and hypocotyl. During floral development *AG* expression was initiated normally in the centre of the floral meristem at stage 3 in *clf-2* mutants and subsequent expression at stage 5 and 8 also mirrored the wild-type pattern of *AG* expression. However from stage 9 onwards ectopic *AG* expression was detected in the petals.

Ectopic *AG* expression occurs only late in floral development demonstrating that *CLF* is not required to specify the initial expression pattern of *AG* in floral meristems but rather to maintain the repression pattern of *AG*. Molecular cloning of *CLF* revealed a structure consistent with such a maintenance function. *CLF* encodes a protein with extensive homology to the product of *E(z)* a *Drosophila* Polycomb gene involved in the maintenance of homeotic gene expression states.

CLF is expressed persistently and ubiquitously during plant development. In the flower *CLF* is expressed from stage 1 in floral meristems. By stages 6 to 9 *CLF* is expressed strongly in the petals, stamen and carpels, and weakly in the sepals. Weak expression is present in all whorls at stage 12. Because *CLF* RNA is present in all four floral whorls but only acts to repress *AG* in whorls 1 and 2, expression of *CLF* alone is insufficient to repress *AG*. *CLF* may interact with domain-restricted factors, to achieve localised effects on its targets.

1.4.5.2 The Fertilisation independent seed development genes

The *Arabidopsis FERTILISATION INDEPENDENT SEED (FIS)* genes act to control cell proliferation (Grossniklaus *et al*, 1998; Ohad *et al*, 1996; Chaudury *et al*, 1997). Two of these genes, *FERTILISATION INDEPENDENT ENDOSPERM (FIE)* and *MEDEA (MEA)* are the respective homologues of *Drosophila Esc* and *Ez*, Pc-G genes in *Drosophila*. *FERTILISATION INDEPENDENT SEED 2 (FIS2)* encodes a Zinc finger transcription factor which interacts with other FIS proteins (Luo *et al*, 1999), suggesting that it may provide a similar function to the zinc finger gap protein Hb of *Drosophila*. All three *fis* mutants confer maternal effects on zygotic (embryo and endosperm) development. In addition *fis* mutants give rise to limited seed development in the absence of fertilisation, although this phenotype has incomplete penetrance.

In plants, gametes are produced from multicellular haploid structures called gametophytes. Pollen grains are the male gametophyte that fertilise the female gametophyte, or embryo sac which resides within the carpels of a flower. In

flowering plants, a double fertilisation occurs, and gives rise to an embryo, and a second zygotic product, the endosperm. One sperm cell fuses with the haploid egg to produce a diploid zygote which gives rise to the embryo. Another sperm cell fertilises the bi-nucleate central cell to create a triploid endosperm, a nutritive source for the embryo. Endosperm, embryo and the surrounding sporophyte must development in a co-ordinated fashion to ensure normal seed development.

1.4.5.3 MEDEA is regulated by imprinting

MEDEA has an earlier role than *CLF*, regulating growth during embryogenesis. Embryo development in the *mea* mutant is characterised by excessive cellular proliferation. Each morphological stage is delayed such that when a wild-type embryo has already reached late torpedo or cotyledonary stage whilst a *mea* embryo lags behind as an abnormally large heart stage embryo, subsequently *mea* seeds abort. Endosperm development in *mea* mutants is also characterised by abnormal proliferation. Sorensen *et al* (2001) have recently demonstrated that the anterior-posterior polar axis of the endosperm is perturbed in all three *fis* mutants.

The *mea* mutant displays gametophytic maternal effect embryo lethality (Grossniklaus *et al* 1998; reviewed in Grossniklaus, 2001). Embryos that inherit a defective maternal *mea* allele invariably abort regardless of whether they recieve a wild-type paternal allele or not. Maternal effects on embryogenesis might result from any of three different situations. Firstly maternal *MEA* products such as proteins or RNA might be deposited in the egg or central cell and required early in zygotic development (as with many *Drosophila* maternal effect genes such as *bicoid* etc).

Secondly the endosperm might be highly sensitive to the dosage of paternal and maternal contributions, so that at least 2 *MEA*$^{+}$ doses are required. The last option is genomic imprinting of the *mea* gene resulting in silencing of the paternal allele during early zygotic development

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Genomic imprinting describes the differential expression of alleles depending on their parental origin. Parent of origin specific differences in gene expression have mainly been described in mammals and are essential for mouse germline development (reviewed in Barlow, 1995). Several maize genes have been identified which are imprinted in the endosperm. The paternally inherited allele has a reduced expression relative to the maternal copy in each case. However these genes are highly polymorphic and imprinting only occurs in a subset of alleles suggesting that the imprinting may not have functional importance.

The parental conflict theory has been proposed to explain why imprinting has evolved despite the vulnerability resulting from the functional haploidy of genes affected (Haig and Westoby, 1991). A parental conflict exists concerning the allocation of resources from mother to offspring in viviparous species. The mother has an equal genetic stake in all of the offspring whereas a father, at least in a polygamous species, may only have stake in some of the embryos and would prefer a greater share of resources to nurture the embryo/s fertilised by him. This could lead to evolution of differential expression, so that genes promoting zygote growth become silenced in maternal alleles and those restricting it are silenced in paternal

alleles. Consistent with this theory, *Arabidopsis* mixed ploidy crosses have demonstrated that by increasing maternal to paternal ratio to 4m:1p endosperm proliferation is restricted. A reciprocal effect occurs when the paternal contribution is increased (Scott *et al*, 1998)

A variety of interploidy crosses were used to demonstrate that the *mea* maternal effect embryo lethality is not caused by dosages effects in the endosperm. Extra copies of wild-type *MEA* were added to both embryo and endosperm in one study (Grossniklaus *et al* 1998), seed abortion was not rescued. Vielle-Calzada *et al* (1999) confirmed that even when an excess of wild-type alleles exist in the endosperm rescue does not result.

RNA expression analysis by *in situ* hybridisation and RT-PCR has shown that *MEA* is expressed both before and after fertilisation (Grossniklaus *et al* 1998, Vielle Calzada *et al* 1999). The *in situ* studies confirmed that despite maternal expression prior to fertilisation the high levels of *MEA* detected at late heart to torpedo stages could only be accounted for if *MEA* was also zygotically transcribed since any maternally deposited RNA would be highly diluted by this stage. Using improved *in situ* hybridisation it was possible to detect dots representing nuclear transcription foci of *MEA* in the large polar nuclei of the central cell prior to fertilisation. The two maternally derived transcription dots present in the binucleate polar cells were not joined by a third transcriptional dot after fertilisation, strongly suggesting that the paternal allele is transcriptionally silenced in the early endosperm and also showing

that *MEA* is zygotically transcribed from the beginning of endosperm development. Vielle-Calzada *et al* (1999) independently confirmed that the paternal allele was not transcribed in both young endosperm and embryo by RT-PCR. They failed to detect paternal *MEA* transcripts in whole seedpods containing seeds with globular stage embryos plus endosperm.

A consensus of research now agrees that the *MEA* gene is imprinted in both endosperm and embryo during early stages of development. How long imprinting persists, especially in the embryo is still debated (reviewed in Grossniklaus *et al*, 2001). However, is *MEA* imprinting biologically relevant to the maternal effects of *mea*? It could be that maternal *mea-* is still lethal even with early paternal expression.

The *mea* phenotype could result either from a lack of maternal loading before fertilisation or because paternal alleles are silent in zygotes early after fertilisation. Partial rescue of the *mea* phenotype in a homozygous *ddm1* background may implicate the latter (Vielle-Calzada, 1999). In *ddm1* (decreased DNA methylation) mutants there is a 70% fall in cytosine methylation, mostly of repetitive sequences (Jeddeloh *et al*, 1999). Since *DDM1* encodes a homologue of an SW1/SNF chromatin remodelling protein the wild-type function of *DDM1* is believed to facilitate the methylation of targets, which may result from the action of a chromatin-remodelling complex which allows access of DNA methyl-transferases.

When embryos with a wild-type paternal MEA allele but mutant maternal *mea*, that would usually abort, were also homozygous for *ddm1* the seed are rescued. Vielle-Calzada *et al* (1999) argue that this is a result of reactivation of the paternal allele as a result of demethylation or some other alteration in chromatin structure.

MEA represents the first plant gene to demonstrate imprinting, which is not allele specific, the implication being that additional important developmental genes may be similarly regulated.

1.4.5.4 The interaction between the *Drosophila* Pc-G proteins E(z) and Esc is conserved in plants and animals

FIE, another *FIS* class gene is the homologue of *Drosophila Extra sex combs (Esc)*, a Pc-G gene. *Esc* has been highly conserved since homologues also exist in humans, mice and *C. elegans*, (Simon *et al*, 1995; Schumacher *et al*, 1998; Seydoux and Strome, 1999). *Esc* is unique amongst Pc-G genes as it is not required continuously during development for its function; instead there is an early critical requirement for *Esc* (Simon *et al*, 1995). Similarly there is an early phenocritical stage when *FIE* is required (Ohad *et al*, 1999).

Esc is a WD-repeat protein. Both *Drosophila* Esc and *Arabidopsis* FIE have seven WD repeats. These are believed to fold into a circular structure with seven blades known as a β propeller (Ohad *et al*, 1999). Another example of WD-repeat protein is the transducin beta subunit (Sondex *et al*, 1996). A common theme amongst WD40 proteins is their involvement in protein-protein interactions. Some have more than

one interacting partner, thus forming a scaffold for the formation of multiprotein complexes.

Plant, vertebrate and invertebrate homologues of Esc and E(z) interact and colocalise *in vivo* (Sewalt *et al*, 1998; Jones *et al* 1998; Tie *et al* 1998; Spillane *et al* 2000; Van Lohuizen *et al* 1998). In all cases the interaction occurs between the WD-repeat domains of the Esc homologues and the N-terminal portion of E(z) and its homologues. The almost complete overlap of Esc and E(z) at chromosomal binding sites and the requirement of E(z) for normal chromosome binding of a number of other Pc-G proteins suggests that this complex may be an essential prerequisite for Pc-G function in general (Tie *et al*, 1998; Rastelli *et al*, 1993).

Interestingly although the N-terminal region serves as the interaction surface in both plants and animals, the sequence has not been conserved. Despite considerable divergence in primary sequence MEA can interact with *Drosophila* Esc and E(z) with FIE in yeast two-hybrid assays (Spillane *et al*, 2000). The interaction of MEA and FIE is likely to be significant *in vivo* since: 1) the two genes confer very similar mutant phenotypes; 2) the expression patterns of *FIE* and *MEA* overlap during gametophyte and early zygotic development; and 3) the FIE and MEA proteins co-localise in the nucleus of transfected plant cells (Spillane *et al*, 2000).

E(z) and Esc are more widely conserved than the other Pc-G proteins. The only Pc-G protein homologues identified in plants are E(z) and Esc and this is also true in

C.elegans where MES2 and MES6 are the E(z)/ Esc homologues and no other Pc-G genes occur in the genome sequences (reviewed in Seydoux and Strome, 1999). The highly conserved nature of the E(z)/Esc complex would suggest that these genes also have important developmental roles in other plant species.

This thesis describes the isolation and characterisation of two *Antirrhinum* Pc-G genes which are homologues of the *Arabidopsis CURLY LEAF* gene, which itself is a homologue of *Drosophila* E(z). Comparing the role of Pc-G genes in such distantly related plant species will help to define the roles of Pc-G genes in the plant kingdom.

1.4.5.5 The targets of plant Pc-G genes

The targets of the Pc-G genes in plants and animals may be distinct based on the known targets of the *Arabidopsis E(z)* homologue *CLF*. The targets of *CLF* are the MADS domain homeotic genes *AG* and *AP3*. The targets of *MEA* and *FIE* are as yet unknown.

The targets of *Drosophila* Pc-G genes are the ANT-C and BX-C homeotic genes, which encode transcription factors characterised by a conserved homeodomain DNA binding motif. Mammals have a homologous set of homeotic genes termed the HOX genes, which also form targets for Pc-G genes. In contrast the targets of *Arabidopsis* CLF, as currently known are instead the MADS domain family of transcription factors.

The MADS domain family is ancient; precursors may even have evolved before the separation of bacterial and eukaryotic lineages (Thiessen, 2000). Three major classes exist in plants and animals. The *ARG80* like genes include Serum Response Factor (SRF), a transcription factor with a gene regulatory role as the end point of MAP kinase pathway (Norman, 1988). This family also include yeast genes involved in the determination of cell type such as *MCM1*. The *MEF2*-like genes form a second family and are involved in muscle specific regulation (Olson *et al*, 1995). These two families have not been identified in plants whereas the third family, the *MIKC*-type have only been found in plants, these resemble the *MEF2*-like type more closely than the *ARG-80* family. Results from the genome project show that *Arabidopsis* contains more than 50 MADS domain genes. Duplication of developmental genes in plants is common making it likely that functional redundancy exists between a number of these factors (Martienssen and Irish, 1999). The MADS box family have diverse functions but the best studied subset are the *abc* floral organ identity genes. These have been studied in a number of species, for example *c* function genes homologous to *AG* and *PLE* have been identified in cucumber, maize and the sweetgum tree (Mena *et al*, 1996; Liu *et al*, 1999; Filipecki *et al*, 1997).

Gene regulatory mechanisms appear to have been well conserved between the plant and animal kingdoms (reviewed in Meyerowitz, 1999). It therefore seems likely that the *CLF* Pc-G gene uses similar mechanisms to animal Pc-G genes to silence its targets. The targets of Pc-G genes however may not have been conserved in the two

kingdoms. It appears that Pc-G genes have been independently recruited to repress the homeotic genes of plants and animals.

2. Materials and Methods

2.1 Plant Materials

2.1.1 Plant stocks

2.1.1.1 *Antirrhinum majus (A. majus)* plant stocks

2.1.1.1.1 Wild-type lines

The wild-type lines JI 75 and JI 98 were routinely used in experiments, both were isolated at the John Innes Institute, Norwich. JI 75 has been used in large scale mutagenesis experiments (Carpenter and Coen, 1990) as has JI 98, which was derived from cross between JI 69 and JI 75. The wild-type line Sippe 50, from the Gatersleben seed collection (Stubbe, 1966), was also grown.

2.1.1.1.2 fistulata (fis)

The recessive mutant *fistulata* (*fis*) originated from the Gatersleben seed collection (Stubbe, 1966), in the Sippe 50 genetic background. The petals of *fis* are stamenoid and the petal lobes are reduced. Plants were grown from the F3 generation of an outcross to JI 75. The F1 seed from the outcross ($P292^{1}$) was a gift from R. Carpenter.

2.1.1.1.3 heptandra-715 (hep)

The *heptandra* (*hep*) mutant was isolated in a transposon mutagenesis experiment by A. Hudson, the genetic background is unknown but must be either JI98 or JI75 as

these were the lines used in transposon mutagenesis screens at the John Innes Institute. The lower petals of *hep* are stamenoid and the petal lobes are reduced (McSteen, 1997). Seed from the F2 generation of a cross between *hep* and JI 98 was grown together with homozygous *hep* (both were gifts from A. Hudson).

2.1.1.1.4 polypetala-704 (poly-704)

The semi-dominant mutant allele *poly-704* arose spontaneously in a cross between JI 69 and JI 75. Plants homozygous for *poly* have flowers with a first whorl of sepals and an indeterminate number of petals. Heterozygous *poly* flowers have one extra whorl of petals between the petals and stamens. A heterozygous *poly* line, $H209^5$ was obtained from R. Carpenter. $H209^5$ was the F1 generation of a cross between heterozygous *poly* ($G229^5$, a selfed line from the original isolate of *poly-704*) and heterozygous *plenaII* in the Sippe 50 background. F2 and F3 generations of the line $H209^5$ were grown to obtain both homozygous and heterozygous *poly* and wild-type plants for Southern blot analysis.

2.1.1.1.5 carnosa

The classical dominant mutant *carnosa (car)* is also from the Gatersleben collection. The mutant is strongly retarded in growth and has small curled leaves and few flowers. An F2 cross was grown (*car*- (Sippe 50) x *CAR*+ (JI 98)).

2.1.1.2 *Arabidopsis thaliana* plant lines

2.1.1.2.1 *AGAMOUS (AG)* reporter lines

The pAG-I::*GUS* reporter construct was obtained from Leslie Sieburth (Sieburth and Meyerowitz, 1997). Both pAG-I::*GUS/ CLF+/CLF+* and AG-I::GUS/*clf-/clf-* lines were grown (see Chapter 4 for more details)

2.1.1.2.2 Steroid inducible CLF+ lines

The dexamethasone inducible lines I36 and I38 were developed by Colin MacDougall and Justin Goodrich. Homozygous *clf 2-/clf 2-* lines were transformed with a glucocorticoid receptor-CLF fusion protein. Homozygous lines were selected using resistance to the herbicide BASTA (see chapter 4 for more details)

2.1.1.2.3 AG-GUS inducible CLF+ lines

The pAG-I::*GUS* reporter line was crossed to I38. In the F2 of the cross , plants that carried the inducible CLF transgene were selected using BASTA herbicide resistance. Resistant plants were tested for possession of the pAG-I::*GUS* reporter by staining flowers in X-Glucuronide (X-Gluc). Screening the F3 progeny of these lines identified F2 plants homozygous for both AG-I::*GUS* and the steroid inducible CLF+ construct (see chapter 4 for more details).

2.1.2 **Plant growth conditions**

2.1.2.1 Seed sterilisation and stratification

Arabidopsis and *Antirrhinum* seeds were sterilised by treating with chlorine vapour in the fume cupboard. The required number of seeds were aliquoted into an

eppendorf tube and placed in a sealed vacuum infiltration chamber. Chlorine gas was produced by the addition of 3ml of concentrated Hydrochloric acid to 100ml of Sodium Hypochlorite. Immediately after this addition the lid was placed on the chamber, bleaching was allowed to continue overnight. All seeds were cold stratified after sowing to synchronise germination. Seeds were imbibed on GM agar plates and placed at 4 °C for 2 to 3 days.

2.1.2.2 Germination and growth of seeds on soil

Arabidopsis seeds were grown on a 3:1 mix of compost and grit, under conditions of constant light. Dexamethasone inducible lines were grown under inductive conditions by watering with a solution of 10 μM dexamethasone every other day. Plants were also regularly sprayed with a solution of 10 μM dexamethasone and 0.001% (v/v) silwet, a surfactant. *Antirrhinum* was germinated and grown on Sinclairs Growers Crop Specific Compost in a greenhouse under long day conditions (16 hours of light and 8 hours of darkness).

2.1.2.3 Growing *Arabidopsis* lines on GM Plates

Seeds were sterilised with chlorine gas and sown on petri dishs containing GM agar containing 200 μg/ml Cefotaxime antibiotic and 20 μg/ml Rovrall fungicide. Steroid inducible clf+ lines were rescued by the addition of 10 μM Dexamethasone (from a 10 mM stock in 100% ethanol) to these plate conditions. The plates were placed in a growth cabinet at 22°C under constant light.

2.1.2.4 Inoculation of *Antirrhinum* with transgenic tobravius

Young wild-type *Antirrhinum* plants (4-6 leaves) were innoculated with transgenic Tobravirus in a growth room at between 20 and 22°C to maximise infection efficiency. Innoculum was thawed and ground in a pestle and mortar with a small amount of tap water. Leaves chosen for inoculation were sprinkled with carborundum powder. Gloved hands were used to rub the virus onto the selected leaves. After a couple of minutes excess carborundum and innoculum was rinsed from inoculated leaves with tap water. All inoculated leaves were covered with newspaper overnight.

2.2 DNA Analysis

2.2.1 Isolation of DNA

2.2.1.1 Isolation of plasmid DNA

Small quantities of plasmid DNA were prepared using a method modified from Ish-Horowitz and Burke (1981). A single colony of host bacteria was inoculated in 5ml of Luria-Bertani (LB) media (10g bactotryptone, 5g bactoyeast extract, 10g NaCl pH 7.0 (per litre)) containing the required antibiotic. This was incubated overnight at 37°C with rigorous shaking. A 1.5 ml aliquot of this culture was transferred to a 1.5ml eppendorf tube and spun at 13,000 rpm on a bench top mini centrifuge for 1 minute to harvest cells. The medium was removed by aspiration and 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0)) was added. The pellet was resuspended by vortexing. To lyse the cells 200 µl of Solution II (0.2 M NaOH, 1% SDS (w/v)) was added. The eppendorf tube was inverted 5 times to mix the contents and then placed on ice for up to 5 minutes. Next, 150 µl of Solution III (100 ml contained 60 ml of potassium acetate (5 M), 11.5 ml glacial acetic acid and 28.5 ml H_2O) was added to precipitate denatured chromosomal DNA and proteins, gentle vortexing was used to disperse the solution. To aid precipitation the sample was placed on ice for 5 minutes. The precipitate formed was removed by spinning for 5 minutes at 13,000 rpm on a mini bench top centrifuge. DNA was precipitated from the supernatant by the addition of two volumes of ethanol. Precipitated DNA was pelleted in a 5 minute spin at 13,000 rpm

on a bench top mini centrifuge and washed using 70% (v/v) ethanol. The DNA pellet was then air dried for 10 minutes and dissolved in 30μl of TE pH 8.0 (10 mM Tris.HCL pH 8.0, 1mM EDTA).

The QIAGEN QIA Spin Miniprep kit was used to extract small quantities of plasmid DNA required for salt sensitive applications such as sequencing. A 1.5ml aliquot from a 5ml overnight culture was spun at 13,000 rpm for a minute. After removal of supernatant, 250 μl of Buffer P1 (containing RNase A) was added and the pellet was resuspended. To lyse the cells 250 μl of Buffer P2 was added. Denatured chromosomal DNA and proteins were precipitated by adding 350 μl of N3 buffer. The precipitate formed after the addition of N3 buffer was pelleted by spinning for 10 minutes at 13,000 rpm. The supernatant was applied to the centre of a QIAprep spin column. This column was washed with PE buffer and the DNA eluted with 30 μl of EB buffer, using a one-minute spin at 13,000 rpm.

Larger quantities of plasmid DNA (up to 100μg) were isolated using the QIAGEN QIAfilter Plasmid Midi protocol. A 5 ml LB culture was incubated at 37°C with the appropriate antibiotic for 8 hours with shaking. This culture was used to inoculate a 25ml culture, which was incubated overnight (12-16 hours). The bacterial cells were harvested by centrifugation at 7500 rpm in a Sorvall SA-600 rotor. The pellet produced was drained by inversion. Buffers P1, P2, P3, QBT and QC were used according to the manufacturers recommendations, along with the QIAfilter cartridge and QIAGEN-tip 100. DNA was eluted using 5ml of buffer QF and precipitated with 3.5 ml of isopropanol. This precipitate was spun immediately at 12000 rpm for 30

minutes at 4°C (in a Sorvall SA600 rotor). The resulting pellet was washed with 2ml of 70% (v/v) ethanol and centrifuged again at 12000 rpm for 10 minutes (in a Sorvall SA-600 rotor). After washing the pellet was air-dried and re-dissolved in 150 μl of TE, pH 8.

2.2.1.2 Extraction of plant genomic DNA

Between 5 and 10 grams of young *Antirrhinum* leaves were removed from plants using scissors and stored at -70°C in silver foil. Leaves frozen in liquid nitrogen were ground in a chilled coffee grinder for 50 seconds. Ground tissue was transferred to a 50 ml polypropylene tube containing 20ml of extraction buffer (100mM Sodium diethyldithiocarbonate, 100mM EDTA pH 8.0, 150 mM NaCl, 15mM Na citrate) and 5ml of 10% SDS (w/v). After rigorous shaking chloroform was added to the top of the tube. The tube was incubated at room temperature for 10 minutes with occasional inversions, then spun for 5 minutes at 3,500 rpm (in a Mistral 1000 centrifuge). The upper aqueous phase was added to a clean 50 ml tube containing 12.5ml of phenol, chloroform was then added to the top of the tube. After 5 minutes of occasional mixing the tube was spun again at 3,500 rpm for 5 minutes (in a Mistral centrifuge). After another chloroform extraction, nucleic acids were precipitated by addition of an equal volume of absolute ethanol to the aqeous phase. The tube was spun for 5 minutes at 3500 rpm in a Mistral centrifuge, the supernatant removed and the pellet spun again for 1 minute to remove all residual ethanol. This pellet was dissolved in 5ml of TE (10mM Tris-HCL, 1mM EDTA, pH8.0) containing 2.5 μl of a 1mg/ml RNase A solution (prepared using Sigma RNase A). The sample was incubated at

37°C for 1 hour. DNA was precipitated by the addition of 0.6 ml of 5M NaCl and 5ml of CTAB (2 % Cetryltrimethylammonium bromide (w/v), 50mM Tris.HCL pH 7.5, 10mM EDTA pH 8.0) and removed by spooling onto a pipette tip. The DNA was washed three times in 70% (v/v) ethanol, 30% (w/v) 0.5M NaCl then air dried and resuspended overnight at 4°C with 0.5ml of TE.

2.2.1.3 Isolation of λ bacteriophage DNA

A 5ml culture of LE392 cells in LB media containing 10 mM $MgSO_4$ was incubated at 37°C overnight with shaking. This was used to inoculate 200ml of LB media (also containing 10mM $MgSO_4$) in a 1 litre flask. Incubation was continued (with shaking) until the optical density at 650 nm (OD_{650}) reached between 0.6 and 0.8. Phage were added to a concentration of 8×10^{10} phage forming units (pfu) from a plate lysate. Incubation was continued until the OD_{650} had fallen to between 0.1 to 0.4, and bacterial lysis had resulted in visible cell clumps. To complete lysis 800μl of chloroform ($CHCl_3$) was added and the flask was shaken for 10 minutes. Bacterial debris were removed by centrifugation at 5000 rpm for 10 minutes in a Sorvall GSA rotor. The lysate was transferred to a fresh container where 400μl of L1 buffer (300 mM NaCl, 100mM Tris-Cl, pH7.5, 10 mM EDTA, 0.2 mg/ml BSA, 20 mg/ml RNase A, 6mg/ml Dnase I) was added. This was placed in a 37°C water bath for at least 30 minutes. To purify λ bacteriophage DNA the Qiagen Midiprep Lamda kit was used according to the manufacturers specifications. This involved using buffers L2, L3, L4 and L5 as described. A tip-100 was used to bind Lambda DNA, this DNA was washed using Buffer QC and eluted with Buffer QF. Following elution the DNA was

precipitated using 3.5 ml of isopropanol. λ DNA was pelleted in a 30 minute 4 °C spin at 12,000 rpm using a Sorvall SA-600 rotor. The pellet was washed with 70% (v/v) ethanol and air-dried. DNA was eluted in TE buffer at pH 8.0.

2.2.2 Restriction digestion of DNA

Genomic DNA (5 μg) was digested in 30 μl reactions containing 20 units of restriction endonuclease and 3 μl of the appropriate 10x buffer. Four different 10X buffers were used each contained 100mM MgCl, 100 μg/ml BSA and 10 mM spermidine together with varied NaCl and Tris concentrations (Table 2.1). The reactions were incubated overnight, the temperature used for incubation was that recommended by the manufacturer of the restriction enzyme, usually 37 °C.

Table 2.1 Restriction digest buffers for plant genomic DNA

Buffer type	NaCl	Tris-HCl pH 7.5	$MgCl_2$	BSA	Spermidine
Low salt	0 mM	100 mM	100 mM	100µg/ ml	10 mM
Medium salt	500 mM	100 mM	100mM	100 µg/ml	10 mM
High salt	1 M	500 mM	100 mM	100 µg/ml	10 mM
Very high salt	1.5 M	500 mM	100 mM	100 µg/ml	10 mM

Plasmid and phage DNA was digested at the desired quantity for a shorter time period (between 1 and 2 hours) using 1X restriction buffer from 10X manufacturers restriction enzyme stocks.

2.2.3 Agarose gel electrophoresis of DNA

Small meniscus gels (15cm x 6 cm) were run to estimate DNA concentration, check PCR reactions and small-scale restriction digests. They were also used for the Southern blotting of transposon mutagenesis PCR screens and RT-PCR. The agarose gel (1% w/v agarose, 0.5x TBE, 10μg/ml ethidium bromide) was poured by pipetting

onto a dry glass plate. After the gel had set it was placed in a tank containing 0.5x TBE. The DNA was run using 1x loading buffer (from a 10X stock containing 25% (w/v) Ficoll, 0.25% (w/v) xylene cyanol and 0.25% (w/v) bromophenol blue) at about 8 Volts/cm for an hour.

Large tanks (21cm x 24cm) were used to pour 325 ml of agarose (0.7% (w/v) agarose, 0.5x TBE) required for genomic digests. The gel was run at 30 Volts/cm for 65 hours and stained with ethidium bromide (10 microgram/ml in TBE) for 30 minutes.

2.2.4 Southern Analysis

2.2.4.1 Southern blotting

After the agarose gel was stained with EtBr and photographed under UV, it was soaked twice for 15 minutes in a solution of 0.25 M HCl, with gentle shaking. Two 15 minute washes with denaturation solution (0.5M NaOH, 1.5M NaCl) followed. Lastly the gel was washed twice for 15 minutes in neutralisation solution (0.5M Tris.HCl pH 7.2, 1.5M NaCl, 1 mM EDTA). Two sheets of Whatmann 3MM paper (21 cm x 50 cm) were soaked in 20x SSC (1M NaCl, 100mM Na Citrate). These were placed on top of a glass plate suspended above a tank containing 20x SSC, so that the ends of the paper acted as a wick. The gel was placed on top of the wet Whatmann paper. A piece of Hybond-N (Amersham) Nylon membrane was cut to

the exact size of the gel, this was soaked in 2 x SSC before being placed on top of the gel. A piece of Whatmann 3MM filter paper also soaked in 2x SSC was placed on top of this. Eight more pieces of Whatmann paper and a stack of paper towels were arranged carefully above this. The whole assembly was weighted and old autoradiographs were placed around the gel to ensure an upward current of 2x SSC. Blotting was allowed to continue overnight. The filter was washed briefly in a solution of 2x SSC and crosslinked using a UV transilluminator at 0.4 J cm^{-2}. The filter was also baked at 80°C for 1 hour.

PCR reactions (including transposon mutagenesis screens) were run on meniscus gels and Southern blotted using a similar method. The gel was washed for 5 minutes in denaturation solution and 5 minutes in neutralisation solution. Then the gel was placed on a flat surface with clingfilm underneath. A 2x SSC soaked Hybond-N nylon filter was placed on top of the gel, blotting paper and tissue were assembled above this. The whole assembly was wrapped in the cling film and this was weighed down and left overnight. Crosslinking was carried out as described above.

2.2.4.2 Radiolabelling of DNA fragments

The DNA fragment (500 ng) was heated for 5 minutes at 100°C with 1 µl of hexanucleotide mix (50 ng/µl) then immediately placed on ice, once chilled it was added to the labelling reaction. The 20µl labelling reaction contained 50 mM Tris.HCl pH 6.9, 10 mM $MgSO_4$, 100 µM dithioreitol, 60 µM dNTP, 60 µM dTTP, 60 µM dGTP, 5 units of DNA Polymerase 1 (Klenow fragment) and 50 µCi α-32P

dCTP. After a 1 hour incubation at room temperature 160 µl TE pH 8 and 20 µl of 10x NT dye (1% Dextran blue, 0.1% Orange G in TE) was added. This reaction was placed over a 1 ml Sephadex G50 column to separate the probe from the unincorporated dNTPs. The blue fraction of the column containing the radiolabelled probe was collected and stored in a freezer.

2.2.4.3 Southern blot analysis using radioactive DNA probes

Prehybridisation was carried out in rotating Duran bottles for 1 hour at 65°C, 200 ml of hybridisation solution was used for a large blot (1g dried milk, 40 ml 20x SSC, 20 ml 10% (w/v) SDS, 140 ml Water). Filters were hybridised overnight at 65°C (high stringency) with 30 ml hybridisation solution containing the denatured radiolabelled DNA probe.

The filter was washed 3 times for 15 minutes, first with 2x SSC, 0.5% (w/v) SDS at room temperature, then 1x SSC, 0.5% (w/v) SDS at room temperature and lastly 0.1x SSC, 0.5% (w/v) SDS preheated to 65 °C. The damp filter was wrapped in Clingfilm and placed in an X ray cassette with an intensifying screen, X ray film (Cronex, Dupont) was exposed at –70°C for 1-3 days.

2.2.4.4 DIG labelling of DNA fragments

Digoxigenin-11-dUTP was incorporated into the DNA during a PCR reaction. The 50 µl reaction (0.32µM Upstream and Downstream primer, 1 unit Taq DNA Polymerase, template DNA (2ng/µl)) included 1x Boehringer PCR Buffer (10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) and 5µl of Boehinger PCR DIG Probe

Synthesis Mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.3 mM dTTP, 0.7 mM alkali-labile DIG-11-dUTP, pH 7.0). PCR was carried out on a DNA Engine, 37 reaction cycles were used.

2.2.4.5 Southern blot analysis using DIG labelled DNA

Blots were prehybridised at 65°C for 2 hours in sealed bags containing 20 ml of hybridisation solution per 100 cm^2 of membrane. The hybridisation solution (5x SSC, 0.1% (w/v) N-Lauroyl-Sarcosine, 0.02% (w/v) SDS, 1% Blocking Reagent (Boehringer)) was made up using a 10% (w/v) stock of Blocking Reagent in Maleic acid buffer (100mM Maleic acid, 150mM NaCl, pH 7.5). Hybridisation was carried out overnight at 65°C using 3.5 ml of fresh hybridisation solution per 100 cm^2 of membrane and 5-25 ng/ml of DIG labelled probe. After hybridization the solution was poured into a sterile container and stored at –20 °C for reuse.

The filters were washed twice, 5 minutes each time (room temperature, gentle shaking) with 2x SSC, 0.1% (w/v) SSC (200 ml per wash for a large blot), then twice for 15 minutes each time with 0.5x SSC, 0.1% (w/v) SDS at 68°C with gentle shaking. Filters were placed in 1x Blocking reagent (5 ml 10x Blocking reagent, 45 ml Maleic acid buffer) for 60 minutes in a flat clean tray with gentle shaking. Anti-Digoxigenin-AP antibody was diluted 1:10,000 in fresh Blocking solution and filters were incubated for 30 minutes, with gentle shaking. The filters were washed twice for 15 minutes in Washing buffer (Maleic acid buffer, 0.3% (v/v) Tween 20). Detection was carried out using a chemiluminescent alkaline phosphatase substrate

(CSPD, supplied by Boehinger-Mannheim). The filters were equilibriated in Detection buffer (100mM Tris-HCl, 100mM NaCl, pH 9.5) for 5 minutes. CSPD was diluted 1:100 with Detection buffer (100µl in 10 ml for a large blot). The filter was placed in a clean flat tray and the CSPD solution dripped over this to saturate the membrane. After 2 minutes of continuous agitation the filter was drained, placed in a sealed bag and incubated at 37 °C for 15 minutes. The filter was then placed in an X-ray cassette, X-ray film was exposed at room temperature for up to 2 hours.

2.2.5 Gel extraction and purification

The DNA was visualised under low level UV and excised from the gel using a clean scalpel. The QIAGEN QIAquick Gel Extraction Kit was used to extract and purify the DNA. Elution of the DNA from the QIAquick column was carried out by adding 30 µl of Buffer EB (10 mM Tris-HCl, pH 8.5), waiting for one minute and then centrifuging for 1 minute.

2.2.6 Preparation of DNA samples for sequencing

Plasmid DNA (0.5 µg) was dried in a vacuum concentrator (Savent). The DNA was redissolved in 4.4 µl of sterile distilled water. Then 1.6 µl of a 1 µM primer stock was added along with 4 µl of dRhodamine terminator mix (PE Applied Biosystems).

The 25 cycles of PCR were as follows; 96 °C for 30 seconds, 96 °C for 30 seconds, 50 °C for 15 seconds, 60 °C for 4 minutes

The DNA was precipitated in a 0.5 ml eppendorf tube by adding 1 µl of 3M NaOAc pH 4 and 25 µl of ethanol. The reaction was placed on ice for 10 minutes and then spun in a desk top mini centrifuge for 15 minutes. The DNA was washed in 70 % (v/v) ethanol and dried on a 37°C heating block. Samples could be stored frozen before sequencing. DNA analysis was carried out using GCG9 and GCG10, sequence analysis packages from the University of Wisconsin (Devereux *et al*, 1984).

2.2.7 Subcloning of DNA fragments

2.2.7.1 Ligation of Lambda cDNA and genomic clones into pBluescript

The gel purified DNA fragments were ligated into linearised pBluescript (Stratagene) using a vector:insert DNA ratio of 1:1. The ligation was carried out in a volume of 10 µl to 15 µl using between 50-200 ng of vector DNA, 1 unit of T4 DNA ligase and 1x ligation buffer. Ligation reactions were placed in a 15°C water bath overnight.

2.2.7.2 Making electrocompetent cells

Cells competent for electro-transformation were made as follows. A 10 ml overnight of DH5 ∝ was used to inoculate 1 litre of LB broth. This was grown with vigorous shaking until an OD_{600} between 0.5-0.7 was achieved. The cells were chilled on ice

water for 30 minutes and spun in a chilled centrifuge at 4,500 rpm (Sorvall GSA rotor) for 15 minutes. All of the supernatant was removed and the pellet carefully resuspended in 1 litre of sterile ice cold 10% (v/v) glycerol. The cells were spun again at 4,500 rpm for 15 minutes in a chilled Sorvall GSA rotor. This step was repeated using first 500 ml of the 10% (v/v) glycerol, then 20 ml and finally resuspended to a final volume of between 2 to 3 mls. Aliquots of 90 μl were prepared and frozen in liquid nitrogen, the cells were then stored at –70 °C.

2.2.7.3 Electro-transformation

Electrocompetent cells were thawed then immediately placed on ice. Competent cells (40 μl per ligation) and 1 μl of ligation reaction were mixed gently with a micropipette in a chilled 1.5 ml eppendorf tube. This reaction was transferred into a chilled cuvette and given a 1.8 kV pulse in a Gene Pulser apparatus set to 200 Ohms and 25 μF. Immediately after shocking the cells were resuspended in 1ml of SOC media (2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).Transformed cells were incubated with shaking at 37 °C for 1 hour. Each ligation was spread onto two selective plates containing X-Gal (40 μg/ml) and IPTG (12.5 μg/ml) and the required antibiotic. The antibiotic was used on the plates to select transformed cells, X-Gal and IPTG to identify those with recombinant plasmid. An aliquot of 100 μl of cells was pipetted onto the first plate, all remaining cells were plated onto the second. White or light blue colonies were selected after an overnight incubation at 37 °C.

2.2.7.4 Cloning of PCR products using the TOPO TA cloning kit (Invitrogen)

A ligation reaction was set up on the bench containing 1 μl of PCR reaction, 1 μl of sterile water and 0.5 μl of pCRII TOPO vector (Invitrogen). The reaction was gently mixed with the pipette and incubated at room temperature for 5 minutes precisely. During this time 2 μl of β-mercaptoethanol was added to 1 tube of thawed One shot competant *E.coli* cells (Invitrogen) on ice, and gently stirred with a pipette tip. After the 5 minute incubation 2 μl of the ligation was added to the cells. The reaction was incubated on ice for 30 minutes. Cells were transformed using a 30 second heat shock in a 42 °C water bath, the reaction was then immediately chilled on ice. After two minutes, 250 μl of room temperature SOC was added and the tube shaken in a 37°C incubator for 1 hour. The transformation was plated on two pre-warmed Kanamycin plates (50 μg/ml) which had been spread with 20 μl of 100 mg/ml X-Gal and 40 μl of 100 mM IPTG. One plate was spread with 100 μl of the transformation, the rest of the cells were plated on the second plate. After an overnight incubation at 37 °C white or light blue colonies were selected, DNA minipreps were made from overnight cultures and analysed by digesting with *Eco* RI.

2.2.8 Polymerase chain reaction

2.2.8.1 PCR cycling conditions

All PCR reactions were carried out on the PTC-200 DNA engine (MJ research) with the exception of PCR screening of transposon mutagenised lines. The primers used in all PCR reactions are shown in Table 2.2. A 'hot start' was performed when there

were small number of reactions. The reactions were preheated to 94°C before Taq Polymerase was added. A 'good start' was used when there were a large number of cycles. Boehringer DNA Taq polymerase was added to the chilled reactions, these were then placed in the PCR machine preheated to 94°C.

All PCR cycling programmes were began with a 2 minute incubation at 94°C. Cycling was then initiated by a 30 second incubation at 94°C. An annealing phase of 30 seconds followed, the temperature of the phase was dependant on the Tm values of the primers used. Annealing was carried out two to three degrees below the lower Tm of the two primers. Products were extended at 72°C, each kilobase of extension required 1 minute at this temperature. Different numbers of cycles were used for different applications. For RT-PCR sub-maximal products were produced using between 25 to 29 cycles, 37 cycles were used for DIG probe synthesis. For standard reactions 35 cycles were used. All products were completely extended using a 7 minute incubation at 72°C, following cycling. The DNA engine was programmed to chill reactions to 4°C after cycling was complete.

2.2.8.2 PCR screening of transposon mutagenised lines

DNA from pools of transposon mutagenised *Antirrhinum* plants were used to screen for transposon insertions in *ANTCLF1* and *2*. Two different screens were used, the large John Innes *Antirrhinum* screen and the Gatersleben mutant screen, both were gifts from E. Keck, E. Coen and R. Carpenter of the John Innes Institute, Norwich. Three different transposon primers were used in combination with *ANTCLF1* and *2*

gene specific primers. The primer Tam 53 (20, Table 2.2) derives from the 5' end of Tam 3, W2052 (21, Table 2.2) from the 3' end. W5667 (19, Table 2.2) was a pre-existing CACTA primer designed from the conserved subterminal region of Tam1, 2, 4 and 5.

PCR reactions were performed using both a gene specific (13-18, Table 2.2) and transposon primers to identify possible insertion sites. The PCR reactions were loaded onto meniscus gels and Southern blotted. Digoxigenin gene specific probes from *ANTCLF1* and *2* were hybridised to the filters to determine whether candidate bands were specific to these genes.

PCR was carried out in a Rapidcycler™ (Idaho Technology) using thin walled capillary tubes. A 10µl volume was used for each reaction (20 µl reactions were sometimes used in confirmatory reactions), this contained PCR buffer (50 mM Tris pH 8.3, 0.25 mg/ml crystalline BSA, 3mM $MgCl_2$, 1mM Tartrazine and 0.5% (w/v) Ficoll 400), 200µM dNTPs, 0.2 µM of gene specific primer, 0.8 µM of transposon primer, 0.4 units of BIOTAQ DNA Polymerase (Bioline) and 1µl of the DNA genomic pool. PCR cycling began with a 4 minute incubation at 94 °C. Annealing was carried out at 50 °C for primer combinations including Tam 3 primers (Tam 53 and W2052), a lower annealing temperature (45°C) was used for reactions including the CACTA transposon primer (W5667) as this primer was degenerate.

Table 2.2 Primers used in PCR reactions (F and R at the end of a primer indicates forward and reverse respectively)

	Name	Oligonucleotide sequence
1	CW1	TCA AAC AGC TGT TGC GCC CA
2	CW2	TTG TCA TGG TCG GGT CTG GA
3	CW3	GCG TGG CAA GAT CTA TGA TC
4	CW4	GGA TAC CAG TCG TTC AGG AA
5	CW5	GAT CTC AGC ACG ATG CAT GT
6	CW6	GCT GAG TTT ACT CAC CAT AT
7	CW7	CCT CCC TGC TCT CTC TTT TG
8	CW8	GGC GCA TCT AAT GAG ATT GA
9	CW9	TGC CCA AGC TGG CGC TCT GT
10	CW1MID	GCT ACT CCT TTT GTC ACT C
11	CW2END	GTG CCA TTG GCG CAT CTA A
12	CW7END	CTC CTT CTG CCA AGC ACT GA
13	G13'IN	TAG GTA ATT TGG AAA GCC GCC
14	G2MIN	CAA TTG TCA TGG TCG GGT CTG G
15	G15'OUT	CTC ATT TGA TGC ACT AGC AGC
16	G15'IN	CCG CTG CTA GTG CAT CAA ATG
17	G2MOUT	CAA CAT CCA GAC CCG ACC ATG A

18	G23'IN	CGC GAC CAT TTG AAG GAG CGC C
19	W5667	GGG ACA TTW ATT TRT GTC
20	TAM 53	CAC GGC CCA ATT CAC ATC TTT A
21	W2052	CTC GGC ACG TTT CAC ATC TTT A
22	DG13'IN	CCT CTC ATC TTT GGT AAC
23	DG2MIN	GAA AAG GAA CCG TGT GGC CC
24	DG2MOUT	CTG GAG GAA ACA CTA GAT CC
25	DG15'IN	TGG CTA CTA GCA ACA CCC
26	DG25'IN	GAC TGA GGA TCA ATC AGT GC
27	CWRAC1	GCC ATA GTT AGA TTG CTC ACT T
28	CWRAC2	GGC CAC CCC CGG TAA AGA T
29	CWRAC3	GCT TCT GGG TTT TCC AGG ACT TT
30	CWRRAC1	CAC CTT GGC AAG ACT TTG AT
31	CWRRAC2	CCA GTG TGG TAT CAG ATA AAC C
32	CWRRAC3	CGT CAA GGC CTT TCT CAA TG
33	CWFRAC1	CTG GAT GTA TTG GCT CGG TAT
34	TUBF	GTT CTT GAT AAC GAG GCC TT
35	TUBR	ACC TTC TTC CTC ATC CTC G
36	YC9	CGG AGC TAG GAG GAG ATT CCT CTC
37	YC10	GCT TGC TCA ACC CAA TTC TGG
38	VG1NCR	ATT CCC AAG AGG TAC CAA GC
39	VG1NCF	TTG AGT TAT GAG CTA GCT G
40	VG2NCR	ATG CGC CAA TGG TAC CCT CA

41	VG2NCF	GAT GCA AAA GAG CTA GCA GG
42	VG1CR	TCT TTG AGA AGG TAC CCC AA
43	VG1CF	GAA CCT TCC TGC TAG CTG TT
44	ANTCLF1RTR	TCA CAA AGC CTC CAT CAG
45	ANTCLF1RTF	GCT ATC TTT GGA CAG AAG
46	ANTCLF2RTR	CAT GAC ATA AAG CAT TGC C
47	ANTCLF2RTF	AAT TGG CAC CAG TAA TGG G
48	AAP (5' RACE)	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG
49	AUAP (5' RACE)	GGC CAC GCG TCG ACT AGT AC

2.3 RNA Analysis

2.3.1 RNA isolation

2.3.1.1 Isolation of total RNA

Arabidopsis seedlings (40 or more young seedlings or equivalent) were ground in a pestle and mortar with 500 μl of RNA extraction buffer (50 mM Tris pH 9.0, 150 mM LiCl, 5 mM EDTA and 5% (w/v) SDS). An equal volume of phenol/chloroform was added to the ground tissue in an eppendorf tube. This was vortexed for 1 minute and then spun for 5 minutes at 13,000 rpm in a desk top minicentrifuge. The aqueous layer was carefully removed and extracted with chloroform by centrifugation as before. After carefully removal of the aqueous layer, 0.2 volumes of 12M LiCl was added and the tube left at 4 °C overnight.

The tube was spun at 13,000 rpm in a mini centrifuge for 10 minutes and the resulting pellet resuspended in 100 μl of DEPC treated water at 80 °C for 2 minutes. A 5 minute spin at 13,000 rpm was used to pellet any undissolved material, the dissolved RNA was removed to a fresh tube. The RNA was re-precipitated by adding 2.5 volumes of ethanol and 1/10 volumes of 3M sodium acetate, the tube was placed at –20 for 1 hour. A 10 minute spin at 13,000 rpm in a mini centrifuge pelleted the RNA, which was washed in 70% (v/v) ethanol (30% DEPC water) and resuspended

in 50 μl of TE pH 8.0. The concentration of the RNA was tested at A_{260}. Purity of the RNA was determined using the ratio A_{260}/A_{280}, RNA was an acceptable purify with a ratio above 1.6.

TRIZOL reagent (Gibco BRL) was used to isolate total RNA from small amounts (100 mg-300 mg) of more mature *Antirrhinum* and *Arabidopsis* leaves. The leaves were ground fresh in a pestle and mortar along with TRIZOL reagent, 1 ml of TRIZOL was used per 50 mg of tissue. The ground tissue was spun at 4 °C for 10 minutes in a mini centrifuge (13,000 rpm) to remove insoluble material. The supernatant was removed to a fresh tube and incubated at room temperature for 5 minutes. Chloroform was added (0.2 ml per 1 ml of TRIZOL originally used) and the tube was shaken vigorously for 15 seconds then incubated again for 5 minutes at room temperature. Centrifugation was carried out at 13,000 rpm for 15 minutes at 4 °C in a mini centrifuge. The resulting upper aqueous layer was carefully pipetted into a new tube.

RNA was precipitated by the addition of 0.5 ml of isopropanol per 1 ml of TRIZOL reagent originally used. After incubation at room temperature for 10 minutes centrifugation was carried out at 13,000 rpm for 10 minutes at 4 ° C in a mini centrifuge. The RNA pellet was washed in 75 % ethanol (1ml per 1ml of TRIZOL reagent used) and spun for 5 minutes at 9,000 rpm at 4 °C. The RNA pellet was air dried for between 5 and 10 minutes and dissolved in DEPC treated water.

2.3.1.2 Isolation of mRNA

Antirrhinum majus inflorescence mRNA for 5' RACE was isolated from total RNA extracted using the QIAGEN RNeasy Plant Mini Kit. The tips of JI75 *Antirrhinum majus* inflorescences were cut off and frozen in foil packets in liquid Nitrogen. One RNeasy Plant Miniprep required 100 mg of tissue, this was ground along with liquid Nitrogen in a chilled mortar. The ground tissue was transferred to a 2 ml eppendorf tube along with the liquid nitrogen, which was allowed to evaporate. Buffer RLT (450 μl) was added immediately after evaporation before thawing began. The protocol was followed and resulting RNA was eluted in the RNase-free water provided. The QIAGEN Oligotex Batch Protocol was used to isolate mRNA. The total RNA (250 μg) was diluted to a 250 μl volume with DEPC treated water. The stated volumes of 2x Binding buffer, Oligotex suspension and Wash buffer OW2 were then used to isolate the poly(A)+ mRNA.

2.3.2 RT PCR

Total RNA (5 μg) in 13 μl of DEPC treated water was heated to 80 °C for 5 minutes, then cooled rapidly on ice. The following components were pipetted together on ice: 4 μl of M-MLV reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl and 50 mM DTT), 1 μl 10 mM dNTPs, 2 μl 0.1M DTT, 0.5 μl of 8 μM QT primer and 0.25 μl RNase inhibitor. The total RNA was added to the tube

along with 1 μl (200 units) of M-MLV Reverse Transcriptase, RNase H minus (Promega).

The reaction was then incubated as follows: room temperature for 10 minutes; 42 °C for 1 hour; 50 °C for 10 minutes; 70 °C for 15 minutes. The cDNA reaction was chilled on ice and a 2μl aliquot diluted in 8μl of sterile distilled water. An RT-PCR reaction was set up using primers designed to amplify the cDNA, 2 μl of the diluted cDNA was used in the reaction. Amplification was carried out using 25 cycles. The PCR reactions were run on a meniscus gel and analysed by Southern blotting.

2.3.3 5' RACE

The Gibco BRL 5' RACE system was used to obtain 1,600 bp of DNA sequence at the 5' end of *ANTCLF*1. Three gene specific primers CWRAC1-3 were designed from *ANTCLF1* to use in the reaction. First strand cDNA synthesis was carried out using 0.5 μg of *Antirrhinum majus* inflorescence polyA+ mRNA. This was mixed in a thin walled 0.5 ml PCR tube with 2.5 μl of 1μM CWRAC1 primer and diluted with DEPC treated water to a volume of 15.5 μl. The mixture was incubated at 70 °C for 10 minutes then chilled on ice. The following components were added; 2.5 μl of 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2.5 μl of 25 mM $MgCl_2$, 1 μl of 10mM dNTP mix and 8.5 μl of 0.1 M DTT .

The reaction was incubated at 42 °C for 1 minute then 1 μl of SUPERSCRIPT II Reverse Transcriptase (200U/μl) was added. The tube was incubated again at 42 °C for 50 minutes, then 70 °C for 15 minutes. After a brief spin (10-20 seconds) the tube was placed at 37 °C and 1 μl of RNase mix was added. After gentle mixing the tube was incubated at 37 °C for 30 minutes then spun down and placed on ice.

A GlassMax DNA Isolation Spin Cartridge (Gibco-BRL) was used to purify the cDNA. The binding solution (6M NaI) was equilibrated to room temperature and 120 μl was added to the first strand reaction. The reaction was transferred to a GlassMax spin cartridge and spun for 20 seconds at 13,000 rpm. The flowthrough was removed and the spin cartridge washed 4 times with chilled 1x wash buffer. Each time the cartridge was spun for 20 seconds at 13,000 rpm. Next the cartridge was washed twice with 400 μl of chilled 70 % ethanol. A final 1 minute spin at 13,000 rpm was carried out after removal of the second 70% ethanol wash. The cDNA was eluted by pipetting 50 μl of sterilised distilled water preheated to 65 °C into the spin cartridge. The spin cartridge was spun for 20 seconds at 13,000 rpm and the cDNA collected in a fresh tube.

A homopolymeric tail was added to the 3' end of the purified cDNA using terminal deoxynucleotidyl transferase (TdT) and dCTP. The following components were mixed together in a thin walled 0.5 ml PCR tube; 6.5 μl DEPC-treated water, 5 μl of 5x tailing buffer, 2.5 μl of 2 mM dCTP and 10 μl of GlassMax purified cDNA. This reaction was incubated at 94 °C for 3 minutes then chilled on ice for a minute. After

the addition of 1 μl of TdT the reaction was incubated at 37 °C for 10 minutes. The TdT was inactivated at 65 °C for 10 minutes and the reaction placed on ice.

The tailed cDNA was amplified in a PCR reaction. The following components were mixed together in a thin walled PCR tube on ice; 5 μl PCR buffer (200 mM Tris-HCl pH 8.4, 500mM KCl), 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 2 μl of 10 μM CWRAC2, 2 μl of 10 μM Abridged Anchor Primer, 5 μl of dC tailed cDNA and 31.5 μl of sterilised distilled water. A 'good start' was achieved by addition of 2.5 units of Taq polymerase to the chilled reaction just before this was transferred to the DNA engine which had been preheated to 94 °C. Thirty PCR cycles were carried out using an annealing temperature of 62 °C and a 2 minute extension time.

A nested PCR reaction was then performed to generate more 5' RACE product. A 5 μl aliquot of the previous PCR reaction was diluted in 495 μl of TE pH 8.0. The following components were mixed together on ice together with 5 μl of the diluted PCR reaction; 5 μl of PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCL), 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 1 μ 10 μM CWRAC3, 1 μl of 10 μM Abridged Universal Amplification Primer (AUAP) and 33.5 μl of sterilised distilled water. A good start was performed after the addition of 2.5 units of Taq Polymerase, again the annealing temperature was 62 °C and the extention time 2 minutes.

The results were analysed on a meniscus gel, 5 μl of the nested reaction was loaded on the gel and two DNA bands (1.0 kb and 1.5 kb) were visible under UV. The

remaining 45 μl of nested reaction was loaded on a gel and the two bands gel purified. Both bands were cloned using the Topo II PCR vector (Invitrogen) and sequenced.

2.3.4 *In Situ* Hybridisation

In situ hybridisation with digoxigenin labelled probes was used to determine the RNA expression pattern of *ANTCLF1* and *ANTCLF2* in *Antirrhinum* inflorescence and vegetative tissue. *In situ* hybridisation of pre-embedded *Antirrhinum* JI 75 inflorescences was carried out at the John Innes. *Antirrhinum* JI 98 was grown to provide young vegetative tissue for embedding. The *in situ* protocols shown below are based on The Non-Radioactive *in situ* protocol for plants from Kathy Bartons laboratory which were used during vegetative *in situ* analysis (http://wisc.edu/genetics/CATG/barton/protocols.html).

2.3.4.1 tissue embedding

JI98 seedlings were fixed in a solution containing 4% (w/v) paraformaldehyde (all manipulations in the fume hood). The paraformaldehyde was dissolved in 400 ml of 1x PBS pH 11.0(0.13M NaCl, 3mM Na_2HPO_4, 3 mM NaH_2PO_4, NaOH pellets to pH to 11.0) which had been heated to 65°C in a microwave. After the paraformaldehyde had dissolved the solution was cooled to 4°C on ice and H_2SO_4

was added to adjust the pH to 7.0. The following detergents were added to improve penetration of fixative into the tissue; 16 ml DMSO, 400 µl Tween, 400 µl Triton X100.

Freshly dissected tissue was arranged in Histoprep plastic grills (Fisher) and placed in the fixative. Penetration was improved by applying a vacuum until the paraformaldehyde boiled, samples were held in this vacuum for 15 minutes. The paraformaldehyde solution was replaced after vacuum infiltration and the samples fixed overnight gently shaking in a cold room (4 °C). Next day the tissue was washed twice for 30 minutes in 1x PBS also gently shaking at 4 °C. The tissue was dehydrated in the following ethanol series (Table 2.3).

Table 2.3 Dehydration of fixed tissue (all steps at 4 °C with gentle shaking)

	Treatment	Time
1	30% ethanol	60 minutes
2	40% ethanol	60 minutes
3	50% ethanol	60 minutes
4	60% ethanol	60 minutes
5	70% ethanol	60 minutes
6	85% ethanol	60 minutes
7	95% ethanol and 0.1% eosin	overnight

Dehydrated tissue was cleared with Histoclear (CellPath) so that wax could be introduced (Table 2.4). Before the first 100 % ethanol/ eosin 0.1% (v/v) wash the tissue was removed from the (plastic grills) and placed in universal bottles.

Table 2.4 Histoclear washes (all steps at room temperature with gentle shaking)

	Treatment	Time
1	100% ethanol and 0.1% eosin	30 minutes
2	100% ethanol and 0.1% eosin	30 minutes
3	100% ethanol and 0.1% eosin	60 minutes
4	100% ethanol and 0.1% eosin	60 minutes
5	25% histoclear, 75% ethanol	30 minutes
6	50% histoclear, 50% ethanol	30 minutes
7	75% histoclear, 25% ethanol	30 minutes
8	100% histoclear	60 minutes
9	100% histoclear	60 minutes

A second treatment of 100% histoclear with the addition of ¼ volume of wax paraplast chips (BDH-Merck) was left overnight without shaking. The chips were melted in a 42°C heating block, a further ¼ volume of chips were then added and melted. After 2 hours at 60°C this wax/histoclear was replaced with freshly melted wax. A 60°C heating block was used to keep the wax molten overnight. The next day the molten wax was changed twice, the first time in the morning and the second late

afternoon. This pattern was repeated for the next two days. On the fifth day in wax the tissue was placed in plastic moulds. A thin layer of wax was poured into a plastic mould placed on a 60°C heating block. The tissue was carefully orientated then the mould was removed from the heating block and a second layer of wax added just as solidification began. To fill the mould a third layer of wax was added just as the second also began to solidify. The mould was floated in a dish of cold water and tipped upside down once the surface began to solidify and indent. Once solidified the moulds were stored at 4°C.

2.3.4.2 Sectioning

The wax mould was cut using a clean razor blade to form a trapezoid. This was melted onto a metal disc then solidified at 4 °C for 10 minutes. More wax was then cut from the sides of the trapezoid, so that 2 mm of wax remained surrounding the tissue. The metal disc was mounted onto the microtome (Leica) such that the longer of the trapezoid faces was aligned both next to and parallel to the blade.

Ribbons of tissue were cut at a thickness of 8 μm . These were arranged on a ProbeOn Plus coated slide (Fisher Biotechnology) and floated with sterile distilled water. The slide was placed on a 42 °C hot plate for between 5-10 minutes until all crinkles had disappeared in the wax. The water was removed using a sterile pipette and tissue paper. Lastly the slide was blotted gently with lens cleaning tissue (Whatman) to remove any remaining water and placed back on the hot plate overnight. Sectioned tissue was stored at 4 °C and used within a fortnight.

2.3.4.3 *In vitro* transcription

RNA probes were transcribed from the non-conserved regions towards the 5' ends of *ANTCLF1* and *2* (Table 2.5), the conserved SET domain and second cysteine rich domain are both at the 3' end of the gene. A PCR product from the *ANTCLFI* cDNA clone pcc2b was cloned into the pCRII TOPO vector. The pNE1 cDNA pbluescript (SK+) clone, which also omits the 3' conserved region, was used to produce RNA probes from *ANTCLF2.* (for details of the cDNA clones of the *ANTCLF* genes see section 3.2).

Table 2.5 *ANTCLF1* and *ANTCLF2* RNA probes (note: G1no3'A and G1no3'B contain the same insert but in opposite orientations)

Probe name	Clone	Vector	Method of linearisation	Length of linearised fragment	Polymerase	In Situ tissue
ANTCLF1A Antisense	G1no3'A	pCRII TOPO	Not I	800 bp	SP6	Floral
ANTCLF1A Sense	G1no3'A	pCRII TOPO	BamHI	800 bp	T7	Floral
ANTCLF2A Antisense	PNE1	pBS SK+	Bgl II	1200 bp	T3	Floral
ANTCLF2A Sense	PNE1	pBS SK+	EcoRI	1600 bp	T7	Floral
ANTCLF1B Antisense	G1no3'B	pCRII TOPO	PCR	800 bp	T7	Veg
ANTCLF1B Sense	G1no3'A	pCRII TOPO	PCR	800 bp	T7	Veg
ANTCLF2B Antisense	PNE1	pBS SK+	PCR	1200 bp	T3	Veg
ANTCLF2B Sense	PNE1	pBS SK+	PCR	700 bp	T7	Veg

Clones were linearised by digestion or PCR. The digestion method involved setting up a restriction reaction with plasmid Qiagen midiprep DNA (10-30 μg). Digestion was carried out for 4 hours and the product diluted in 300 μl of TE. A QIAGEN QIAquick PCR purification kit was used to purify the linearised product. The PCR approach required using two primers to amplify a fragment containing the gene specific insert as well as the correct polymerase site for *in-vitro* transcription. The PCR products were also purified using the QIAGEN QIAquick PCR purification kit.

Linearised product (0.5-1 μg) was dried in a vacuum concentrator (Savant) and diluted in 11 μl of DEPC treated water. This was added to the following reaction mix; 2.5 μl 10x RNA polymerase buffer (T7 or T3 specific), 0.5 μl RNase inhibitor (20 units), 2.5 μl of 5mM ATP, GTP and CTP, 2.5 μl of 1 mM Dig-UTP and 1 μl of RNA polymerase (T7 or T3) (20 units). After incubated at 37°C for 2 hours the following was added; 75 μl of DEPC treated water, 1 μl of 100 mg/ml tRNA and 0.5 μl of DNase (5 units). Again the reaction was incubated at 37°C. After 15 minutes the RNA was precipitated using an equal volume of NH_4OAc (3.5M) and 2 volumes of ice cold ethanol, then left overnight at –20°C. The RNA was spun down at 13,000 rpm for 10 minutes and washed in 70% ethanol (DEPC treated). The RNA was air dried for 10 minutes and then resuspended in 100 μl of DEPC treated water and 100 μl of 2x carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2). Hydrolysis was carried out at 60°C for the specified time Empirical times suggested in the John Innes in situ protocol were used (Table 2.6)

Table 2.6 Hydrolysis times for *in situ* probes

Length of linearised template (base pairs)	Hydrolysis at 60 °C (minutes)
700	80
800-900	85
1000-1600	90

(modified from John Innes In situ protocol)

The hydrolysis product was precipitated at –20°C for 2 hours and pelleted again at 13,000 rpm for 10 minutes. The RNA was air dried for 10 minutes, resuspended in 50 μl of TE pH 8.0 (DEPC treated) and stored at –70 °C.

The yield from each *in-vitro* transcription reaction was estimated in a spot test using a 1/100 (10 μg/ μl) DIG-labelled control. A 1 μl spot of each newly labelled RNA probe and the 1/100 DIG-labelled control DNA was fixed on a nylon filter by UV crosslinking at 0.4 J cm^{-2}. The filter was washed briefly in Buffer 1 (100 mM Tris pH 7.5, 150 mM NaCl), then shaken gently in Buffer 2 (1x Boehringer blocking reagent in Buffer 1) for 30 minutes. After another brief wash in Buffer 1 the filter was washed again with gentle shaking in 5 ml of Buffer 1 in which 1 μl of anti-DIG antibody had been added. Unbound antibody was removed using two 15 minute washes in Buffer 1. The filter was briefly washed in Buffer 5 (100 mM Tris pH 9.5,

100mM NaCl, 50 mM $MgCl_2$) and placed in Western Blue substrate (Promega) until the purple spots became visible (5 to 10 minutes). Comparison of intensities gave an estimate of the success of the transcription reaction.

2.3.4.4 Section pre-treatment

Slides containing sectioned tissue were placed in a baked metal slide rack that held a maximum of 24 slides. The slides were pre-treated as shown in (Table 2.7) all treatments were in RNase free plastic tubs (a glass tub was used in the first 100% ethanol step) containing 300 ml of the solutions (600 mls were used in step 16). Every step was at room temperature except Proteinase K (step 12) which was performed at 37 °C. A stir bar was used in step 16 (Acetic anhydride) the slide rack was elevated above this. Acetic anhydride was added to the triethanolamine just before the slide rack was placed in the solution. After step 19 the slides could be kept for several hours in a plastic tub containing a small amount of ethanol at 4°C.

Table 2.7 Section pretreatment

	Treatment	Time
1	Histoclear	10 minutes
2	Histoclear	10 minutes
3	100% ethanol	2 minutes
4	100% ethanol	2 minutes
5	95% ethanol	2 minutes
6	90% ethanol	2 minutes
7	80% ethanol	2 minutes
8	60% ethanol	2 minutes
9	30% ethanol	2 minutes
10	DEPC treated water	2 minutes
11	2x SSC	20 minutes
12	1 μg/ml Proteinase K in 100 mM Tris pH 8.0, 50 mM EDTA	30 minutes
13	2 mg/ml glycine in PBS	2 minutes
14	PBS	2 minutes

15	PBS	2 minutes
16	Acetic anhydride (0.5% v/v in 0.1 M triethanolamine pH 8.0)	10 minutes
17	PBS	5 minutes
18	PBS	5 minutes
19	Dehydrated back through ethanol series step 9 to step 3	30 seconds each step

2.3.4.5 *In situ* hybridisation

Hybridisation was achieved by sandwiching together the Probe-On plus slides after a mixture of probe and hybridisation solution had been evenly applied. Excess hybridisation solution for the number of slide pairs was prepared since this solution was very viscous and difficult to pipette accurately. Hybridisation solution for 5 slide pairs comprised 100 μl 10x *in situ* salts (3 M NaCl, 100 mM Tris (pH 8), 100 mM $NaPO_4$ (pH 6.8), 50 mM EDTA), 400 μl deionized formamide, 200 μl 50% dextran sulfate, 20 μl 50x Denhardts solution, 10 μl tRNA (100 mg/ml) and 70 μl DEPC treated water. The hybridisation solution was heated on a 70 °C heating block before use to minimise problems caused by high viscosity.

The pre-treated *in situ* slides were dried completely on clean paper towels prior to hybridisation. RNA probes were thawed on ice and a 1 μl aliquot was diluted in 19 μl of DEPC treated water. This diluted probe was added to 20 μl of formamide. The probe was then denatured at 80°C for two minutes, cooled on ice, spun down briefly and left on ice. Hybridisation solution (160 μl) was carefully added and mixed with each 40 μl preparation of denatured 50% formamide RNA probe. Each 200 μl hybridisation mixture was added to the centre of one of the two slides of a probe pair. The second slide of the pair was carefully sandwiched on top of this enabling the hybridisation mixture to completely cover the inner surface of both slides, avoiding bubbles if possible. Slides were elevated on top of glass rods above 50% formamide soaked paper towels in a square plastic tray sealed using clingfilm and hybridised at 55°C overnight.

2.3.4.6 *in situ* post-hybridisation

All slide pairs were separated and rinsed using a 0.2x SSC solution prewarmed to 55 °C and placed in a slide rack. The following post hybridisation treatments were carried out (Table 2.8).

Table 2.8 *in situ* post hybridisation treatments (all steps with gentle agitation)

	Treatment	Temperature	Time
1	0.2x SSC	Room temperature	60 minutes
2	0.2x SSC	Room temperature	60 minutes
3	NTE	37 ° C	5 minutes
4	NTE	37 ° C	5 minutes
5	20 μg/ml RNAse in NTE	37 ° C	30 minutes
6	NTE	37 ° C	5 minutes
7	NTE	37 ° C	5 minutes
8	0.2x SSC	55 ° C	60 minutes
9	PBS	Room temperature	5 minutes
10	1% Boehringer block in 1x Buffer 1 (100 mM Tris pH 7.5, 150 mM NaCl)	Room temperature	45 minutes
11	1% BSA,100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100	Room temperature	45 minutes

Anti-dig antibody was diluted 1:1250 in 1% BSA, 100mM Tris pH 7.5, 150 mM NaCl, 0.3% (v/v) Triton X-100. Slides were sandwiched together in the original pairs and capillary action used to draw the antibody solution over the inner tissue surfaces

of each pair. This first application was drained onto a clean tissue and the process repeated avoiding bubbles. Slide pairs containing the second application of antibody solution were placed on glass rods in a tray containing wet towels at room temperature. After 2 hours the antibody solution was drained from the slide pairs using clean tissue. Separated pairs were placed in the slide rack and washed 4 times for 15 minutes each in Washing buffer (1% BSA, 100 mM Tris pH 7.5, 150 mM NaCl, 0.3% (v/v) Triton X-100), with gentle rocking. The slide rack was then placed in Detection buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 minutes. To ensure the removal of all detergent each slide was then rinsed separately in this solution. Again slide pairs were sandwiched and capillary action was used to draw Western blue (Promega) solution between the slides. Slides containing the second capillary application of Western blue were placed above wet paper towels, in a plastic dish sealed with clingfilm. The dish was stored in the dark for 2-3 days. To end the colour reaction slides were drained, separated and placed in TE pH 8.0 for 15 minutes. Slides were placed in 0.1% (w/v) calcoflour for 10 minutes to counter stain the cell walls. The calcaflour was rinsed off by dipping the slides in 1 litre of water. Slides were air dried overnight on dry tissue paper. Coverslips (Chance Propper Ltd, No.0, 22x64 mm) were mounted on top of each slide using a few drops of Entellan (Merck).

2.3.5 Histochemical staining to assay β-glucuronidase (GUS) enzyme activity

The GUS gene encodes for the *E.coli* β-glucuronidase enzyme. The colourless X-gluc substrate is cleaved by GUS to give a blue derivative. GUS staining solution contained the active component X-Gluc (5-Bromo-4-Chloro-3-Indolyl-ß-D-Glucuronide). This was dissolved in dimethyfomamide to a concentration of 2mM. A 10 ml stock of the solution contained 684 μl of 0.5M Na_2HPO_4, 316 μl of 0.5M NaH_2PO_4, 50 μl of $K_2Fe(CN)_6$, 50 μl of $K_4Fe(CN)_6$, 5 μl of Triton-X-100 and 200 μl of 2mM X-Gluc in dimethylformamide.

Seedlings were uprooted from soil or plates and placed in the GUS staining solution. The samples were vacuum infiltrated for 5 minutes to improve penetration of the solution. The tissue was stained during a 16 hour incubation at 37 °C. The reaction was stopped by removing the staining solution and replacing this with 70% ethanol. Tubes were shaken gently on a tilting apparatus until the chlorophyll and other pigments had been removed by the ethanol.

3. Characterisation of Polycomb-group genes from *Antirrhinum majus*

3.1 Introduction

The *Arabidopsis CURLY LEAF (CLF)* gene encodes a Polycomb-group (Pc-G) protein that is required to repress the *c* function floral homeotic gene *AGAMOUS (AG)* in the leaves, inflorescence stem and flowers. To test whether *CLF* has similar functions in other plants, or has different targets, *CLF* homologues were characterised from *Antirrhinum majus. Antirrhinum* is distantly related to *Arabidopsis thaliana* thus providing an important comparative system for the study of plant Pc-G function. Unlike animal Pc-G genes, which have many targets, *Arabidopsis CLF* has only one main target, *AG*. Characterisation of distantly related plant Pc-G genes would determine whether, in general, plant Pc-G genes also have few targets like *CLF*, or are promiscuous like animal Pc-G genes.

Antirrhinum has zygomorphic flowers whereas the flowers of *Arabidopsis* are actinomorphic. The interaction between Pc-G genes and those controlling dorsoventral patterning of the flower can therefore be analysed in *Antirrhinum* but not *Arabidopsis. Antirrhinum* was a convenient plant to use for comparative study, since the orthologues of many of the *Arabidopsis* floral homeotic *abc* function genes have also been characterised from *Antirrhinum*. In addition *Antirrhinum* has active

transposable elements which can be used in both forward and reverse genetic screens.

This chapter describes the isolation and characterisation of two *Antirrhinum CLF* homologues named *ANTIRRHINUM CLF 1 (ANTCLF1)* and *ANTIRRHINUM CLF 2 (ANTCLF2)*. To determine the biological function of these genes, their expression patterns were characterised, and screens for mutations were made: firstly transposon mutagenised populations were screened for insertional mutations; secondly classical mutations conferring altered *c* function expression were characterised; and lastly viral induced gene silencing was attempted to reduce or eliminate expression of the two genes.

3.2 Isolation of two *Antirrhinum majus CLF* homologues

Antirrhinum cDNA and genomic libraries were screened at low stringency (55°C hybridisation, 2 x SSC wash) with an *Arabidopsis CLF* probe (encoding the highly conserved SET domain) by R.Waites, to identify *Antirrhinum CLF* homologues. The probe was designed from the conserved portion of the gene and used to screen both a floral cDNA library (in the lambda GT10 vector; prepared by R. Simon) and floral genomic library (in the lambda EMBL3 vector; from Hans Sommer). Five genomic and five cDNA clones were isolated. Restriction analysis and Southern blot analysis suggested that the clones were derived from two genes. To confirm this, the cDNA inserts were subcloned into pBluescript and sequence analysis confirmed that two genes had been identified.

The two *Antirrhinum CLF* homologues were named *ANTIRRHINUM CLF 1 (ANTCLF1)* and *ANTIRRHINUM CLF 2 (ANTCLF2).* Two different cDNA clones were obtained for *ANTCLF2,* pNE3 and pcc4. pNE3 was ~2.4 kb and by digestion using *Kpn* I produced two smaller clones, pNE1 (1.6 kb of 5' sequence) and pNE2 (1 kb of 3' sequence).The longest cDNA clone obtained for *ANTCLF2* was ~3.1 kb (pcc4). *Arabidopsis CLF* produces a transcript of a similar size (3.2 kb) suggesting that the clone was full length. Furthermore, in-frame stop codons were identified in

the 5' UTR upstream of the presumed ATG start codon, confirming that the cDNA contained the entire coding sequence. In contrast, the longest *ANTCLF1* cDNA clone obtained was ~1.7 kb (pcc2b) and sequencing indicated that it was not full length but derived from the 3' end of the gene. 5' RACE (Rapid Amplification of cDNA Ends) was used to isolate the 5' end of *ANTCLF1*. Total RNA and mRNA, isolated from inflorescence tips of *Antirrhinum* wild-type JI 98, was used in the procedure. Two PCR products of ~1.6 kb were obtained, one from the original mRNA reaction and one from the original total RNA reaction. Sequence analysis confirmed that both fragments contained the 5' end of *ANTCLF1*. The fragment derived from the total RNA reaction was slightly longer and included the complete 5' coding sequence and 5' UTR sequence.

The cDNA sequences of *ANTCLF1* and *2* both contained a long open reading frame (ORF) starting at position 150 and 218 respectively. Translation begins at an ATG codon, the translational start site partially conforms to the consensus of Kozak (1987) which is CCA/GCCATGG. *ANTCLF2* has a G at position –3 (3 nucleotides upstream of the ATG) and a C in position –2, whereas *ANTCLF1* does not conform to the consensus at position –3 (having a C instead of an A or G at this position) but does have a C at positions –1 and –2. The first 7 amino acids of the coding sequence are MSAPKAS, a motif conserved between the two genes. The ORF of *ANTCLF1* showed the potential to encode a protein, ANTCLF1, of 890 amino acids (Figure 3.1). The *ANTCLF2* ORF showed potential to encode a protein of 888 amino acids (Figure 3.2). Sequence comparisons using BLAST revealed that both proteins shared

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ATGTCTGCGCCCAAAGCTTCCACTTCCCGGTCCGATCGCCAGGTTGCTGGGCGGCAAGAA
M  S  A  P  K  A  S  T  S  R  S  D  R  Q  V  A  G  R  Q  E     20

ACAAGACGGTCAAAGCAGAAAATCTCAGCAGTCATTAATTCTTTTAGTAAAAAATTTGCT
T  R  R  S  K  Q  K  I  S  A  V  I  N  S  F  S  K  K  F  A     40

AGTGACAGCTCTTCTTATATAGAGAAACGTATGGTAGAGAACATTGAGAAAGGCCTTGAC
S  D  S  S  S  Y  I  E  K  R  M  V  E  N  I  E  K  G  L  D     60

GTTGCAAAAAATCTGTATAAGCTATCTTTGGACAGAAGAAATTTTAAGATTATCGGTGAT
V  A  K  N  L  Y  K  L  S  L  D  R  R  N  F  K  I  I  G  D     80

GATAGAAGTATAGATTTACTTTCGAAGAGACAGAAGGATGCAGTTGATATGCATAATGAT
D  R  S  I  D  L  L  S  K  R  Q  K  D  A  V  D  M  H  N  D    100

ATTGCCACCAAAAGCGAAGATACTGAAAGCAGTAGCTCCCGTGAAGATGGATATGCCTCT
I  A  T  K  S  E  D  T  E  S  S  S  S  R  E  D  G  Y  A  S    120

TCAGCAGTTCTTTTAGGAGCAAGTATTACAGTCAAGAATGCCGTCCTTCCTATAAACCTG
S  A  V  L  L  G  A  S  I  T  V  K  N  A  V  L  P  I  N  L    140

CCAGAAGTGCAAAGATTACCACCATATACATCATGGATCTTTTTGGATAGAAATCAGAGA
P  E  V  Q  R  L  P  P  Y  T  S  W  I  F  L  D  R  N  Q  R    160

ATGCCAGAGGATCAGTCAGTGGTTGGGAGAAAGAGAATCTATTATGACCAGAATGGAGGT
M  P  E  D  Q  S  V  V  G  R  K  R  I  Y  Y  D  Q  N  G  G    180

GAAGCTCTTATTTGCAGTGATAGTGAAGAAGAAGCAATTGATGATGAAGACTCTAAAAGA
E  A  L  I  C  S  D  S  E  E  E  A  I  D  D  E  D  S  K  R    200

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V  F  G  E  S  E  D  Y  I  L  R  M  T  I  E  E  V  G  L  S    220

GATACCACACTGGATGTATTGGCTCGGTATCTATGTAGAAAATCAAGTGAAATCAAGGGA
D  T  T  L  D  V  L  A  R  Y  L  C  R  K  S  S  E  I  K  G    240

AGATATGAAGATCTAGTCAAGAGTGGGGAAGCTATTTATCTTGTAAACCATGGAACTGAT
R  Y  E  D  L  V  K  S  G  E  A  I  Y  L  V  N  H  G  T  D    260

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G  G  F  V  N  P  H  I  N  Q  D  L  D  A  A  L  D  S  F  D    280

AATTGGTTTTGTCGTCGGTGTTTTGTTTTTGATTGTAAATTACACGGGTGCTCACAGGAT
N  W  F  C  R  R  C  F  V  F  D  C  K  L  H  G  C  S  Q  D    300
                    1st cysteine-rich domain
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L  V  F  P  S  E  K  Q  S  S  W  S  C  L  D  V  E  K  N  P    320

TGTGGCCCAGATTGCCATAGACTGGTTCTTAATGCAGAGACTAAGAACAAATTGAGTTAT
C  G  P  D  C  H  R  L  V  L  N  A  E  T  K  N  K  L  S  Y    340

GAGCAAGCTGATTTGGAAGAAAAAGATTCCCCTGCATCCACTGTTACTGCTTCAGATATG
E  Q  A  D  L  E  E  K  D  S  P  A  S  T  V  T  A  S  D  M    360

CATAAGATGAAGAAATCAAAGTCTTGCCAAGGTGAAAGCTCTTCTTCCTCAGTTATCAAA
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AAAAGGAGTAGCAAGAGAGTAGCTGATAATGTTCTAGTTGCCAAACAGAAGAGGCAGAAG
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GGGAATTCAAGTCAAGAAGAAGAAGATATCAGTACTTCCCTGAAAGTGAAATATGAAAGT
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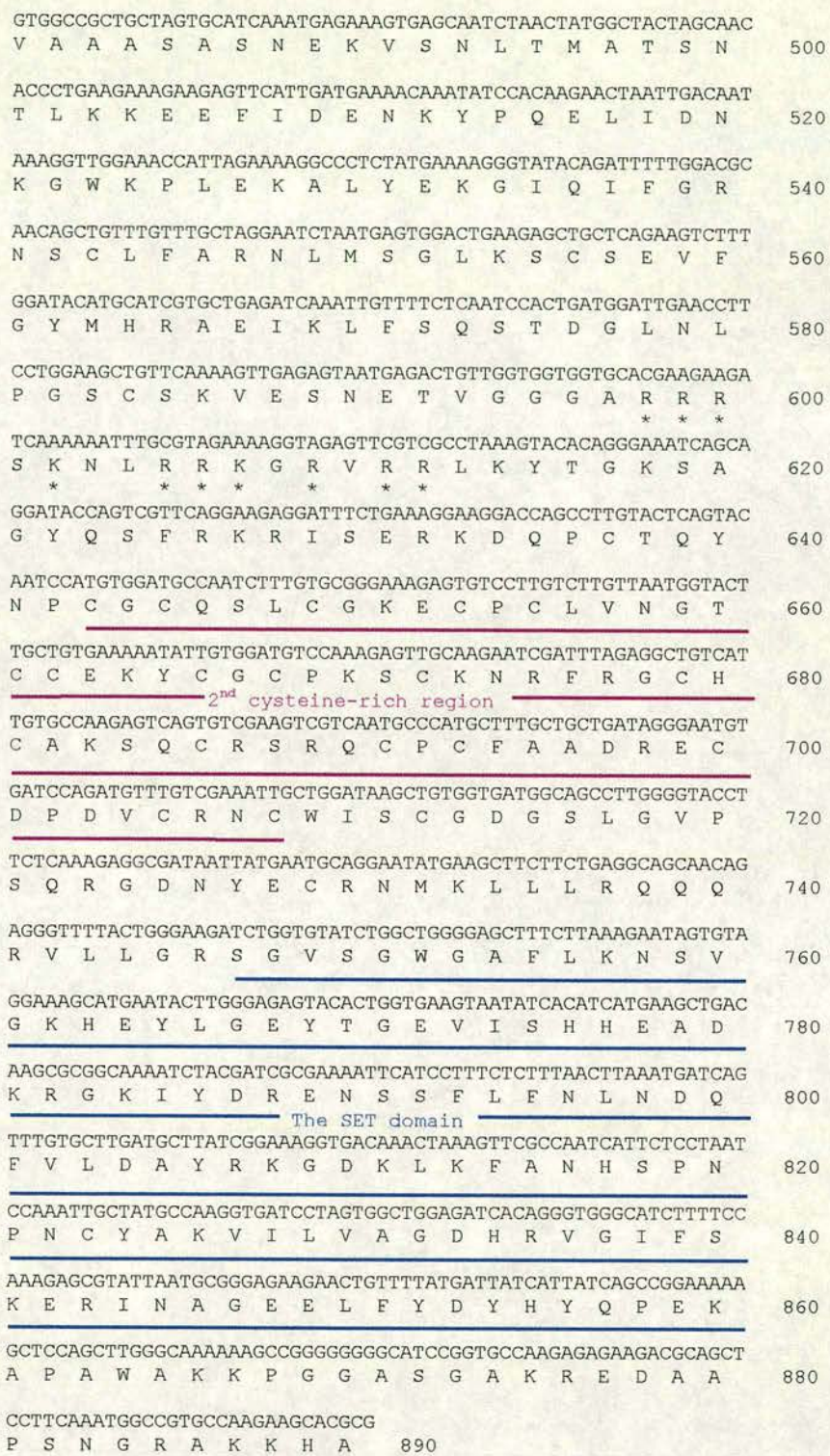
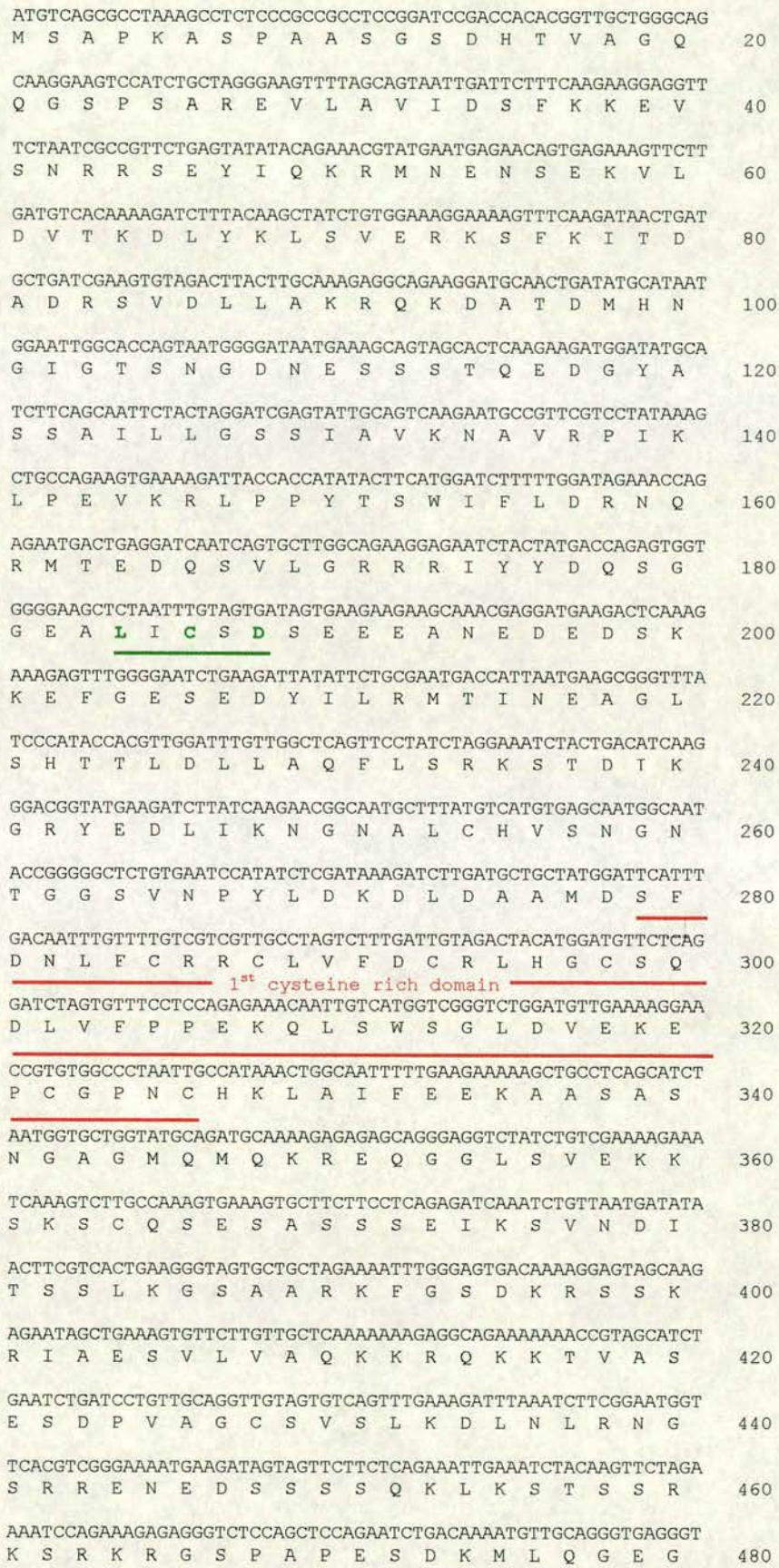


Figure 3.1 The amino acid sequence of *ANTCLF1*, shown below the nucleotide sequence the conserved SET and cysteine rich domains are underlined. Basic residues of a putative bipartite nuclear localisation signal are indicated as asterisks below the amino acid symbol. A putative binding motif for the Retinoblastoma protein (Rb) is indicated (Three green amino acids).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Q G S P S A R E V L A V I D S F K K E V 40
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S N R R S E Y I Q K R M N E N S E K V L 60
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D V T K D L Y K L S V E R K S F K I T D 80
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A D R S V D L L A K R Q K D A T D M H N 100
GGAATTGGCACCAGTAATGGGGATAATGAAAGCAGTAGCACTCAAGAAGATGGATATGCA
G I G T S N G D N E S S S T Q E D G Y A 120
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S S A I L L G S S I A V K N A V R P I K 140
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T G G S V N P Y L D K D L D A A M D S F 280
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D N L F C R R C L V F D C R L H G C S Q 300
1st cysteine rich domain
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D L V F P P E K Q L S W S G L D V E K E 320
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P C G P N C H K L A I F E E K A A S A S 340
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N G A G M Q M Q K R E Q G G L S V E K K 360
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S K S C Q S E S A S S S E I K S V N D I 380
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T S S L K G S A A R K F G S D K R S S K 400
AGAATAGCTGAAAGTGTTCTTGTTGCTCAAAAAAAGAGGCAGAAAAAAACCGTAGCATCT
R I A E S V L V A Q K K R Q K K T V A S 420
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E S D P V A G C S V S L K D L N L R N G 440
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S R R E N E D S S S S S Q K L K S T S S R 460
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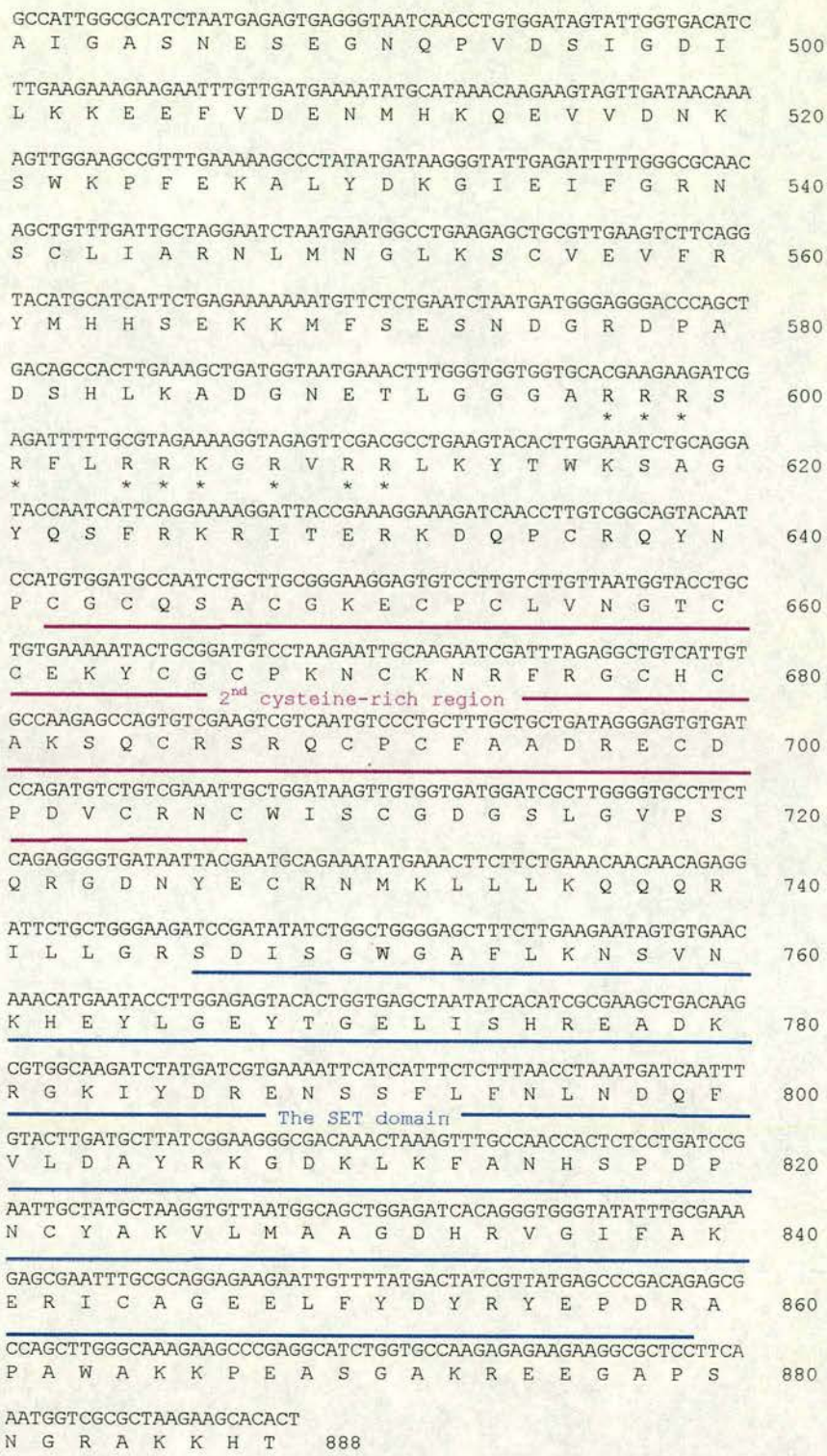


Figure 3.2 The amino acid sequence of *ANTCLF2*, shown below the nucleotide sequence the conserved SET and cysteine rich domains are underlined. Basic residues of a putative bipartite nuclear localisation signal are indicated as asterisks below the amino acid symbol. A putative binding motif for the Retinoblastoma protein (Rb) is indicated (Three green amino acids).

greater homology with CLF than with any other plant E(z) homologues such as MEDEA (Grossniklaus *et al*, 1998), or other SET domain proteins on the database. ANTCLF1 and 2 are, however, more similar to each other (73% amino acid identity) than either is to CLF (58% and 62% amino acid identity respectively) suggesting ANTCLF1 and 2 were duplicated more recently than the *Arabidopsis* and *Antirrhinum* lineages separated.

ANTCLF1 and 2 share most similarity to CLF in the carboxy terminal SET domain and the second cysteine-rich domain (Figure 3.3b). The first cysteine-rich region of ANTCLF1 (residues 278-325) and ANTCLF2 (residues 279-326) is moderately well conserved and contains the five conserved cysteines present in E(z) (residues 321-367). The second cysteine rich region of ANTCLF1 (residues 643-708) and ANTCLF2 (residues 642-707) has been very highly conserved, sharing 92% identity with CLF (Figure 3.3b). There are only 2 amino acid residue changes between ANTCLF1 and 2 within this domain, again suggesting that these genes were produced from recent gene duplication (Figure 3.3a).

There are three other regions of similarity shared between the ANTCLF proteins and CLF (Figure 3.3b). Near the amino-terminus of ANTCLF1 and 2 is a region of 53 amino acids that shares 87% and 89% identify, respectively, with CLF. This region contains a putative Retinoblastoma (Rb) protein binding site (LXCXD) (indicated in Figures 3.1 and 3.2), that has also been identified in CLF (Williams and Grafi, 2000). The putitive bipartite nuclear localization signal present on the amino flank of the

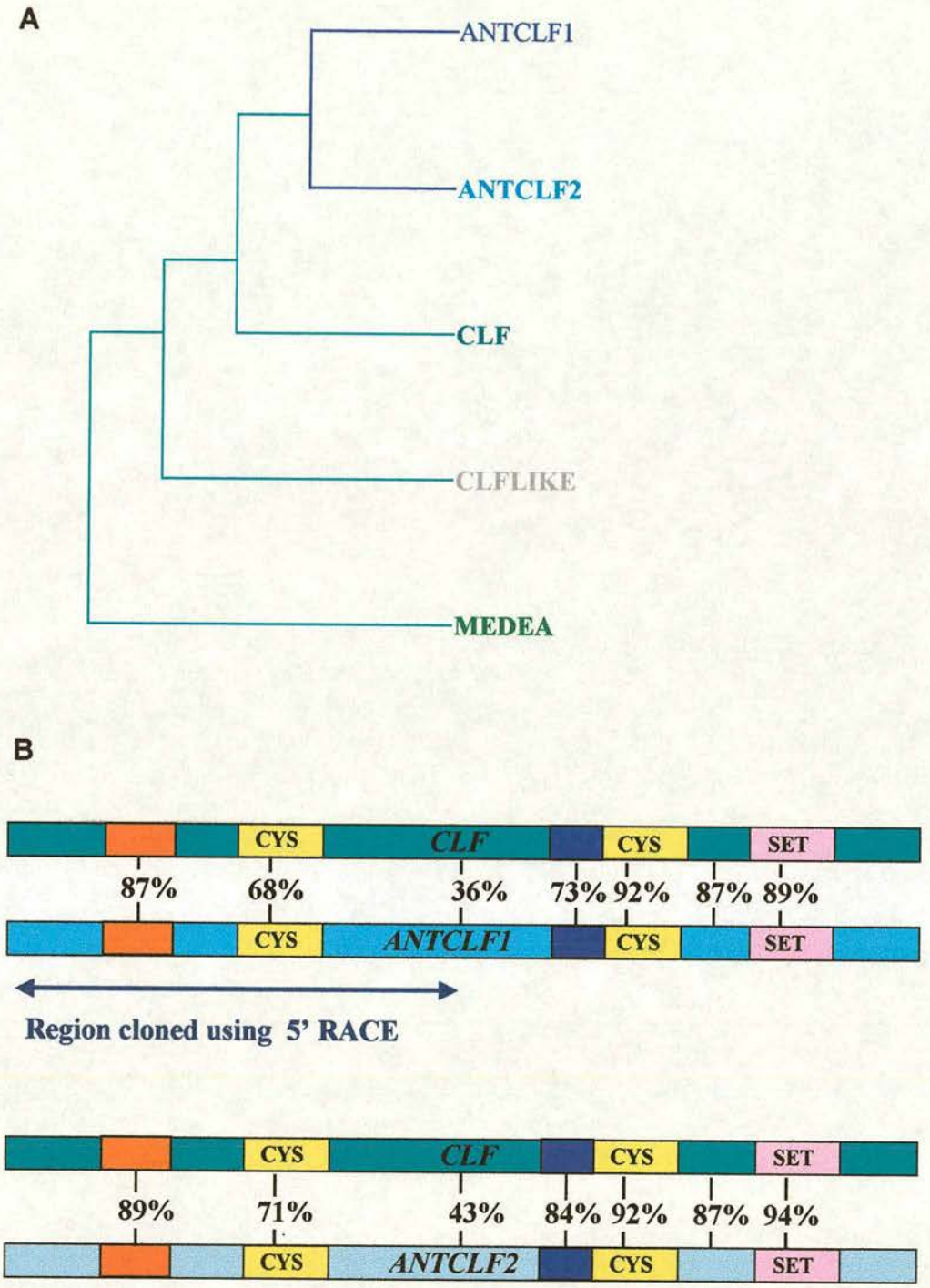


Figure 3.3 *ANTCLF1* and *2* (*Antirrhinum*) show extensive homology to *CLF* (*Arabidopsis*) but are most similar to each other and likely arose from a recent gene duplication.

A) Phylogenetic analysis compiled from the second cysteine rich domain from plant Polycomb-group proteins using the GCG program pile-up.

B) Schematic alignment of CLF with the protein sequence of ANTCLF1 and 2, figures indicate the % amino acid identity within conserved domains. The red region contains a putative Retinoblastoma (Rb) binding motif and the dark blue region contains a putative bipartite nuclear localisation signal. A less conserved region between the 1st cysteine-rich domain and the nuclear localisation domain is indicated.

second cysteine-rich domain in CLF is also present in ANTCLF1 and 2. The domain consists of 10 basic residues arranged in two clusters (see asterisks, Figures 3.1 and 3.2). The nuclear localisation signal is found within a region of high amino acid identity (Figure 3.3b). The third additional region of high identity is located between the 2nd cysteine rich domain and the SET domain (Figure 3.3b).

The carboxy terminal 114 amino-acid SET domain of CLF is highly conserved in ANTCLF1 and 2. SET domain proteins have been classified into four subgroups according to sequence identities within the SET domain (Laible *et al*, 1997). ANTCLF1, ANTCLF2 and CLF fall into the subgroup containing E(z) related proteins. Two additional plant E(z) homologues MEDEA (MEA) and CURLY LEAF-LIKE (CLFLIKE) (a predicted protein of unknown function identified in an *Arabidopsis* sequence database search; J.Goodrich personal communication) are also plant members of this sub-group. ANTCLF1 showed 88% amino acid identity to CLF within the SET domain whilst ANTCLF2 shared 93% identity to CLF within the domain (Figure 3.3b). Multiple sequence alignments (Prettybox, GCG10; Devereux *et al*, 1984) produced from the SET domains of *Arabidopsis* and *Antirrhinum* E(z) homologues and *Drosophila* E(z) demonstrates the high conservation of this domain (Figure 3.4).

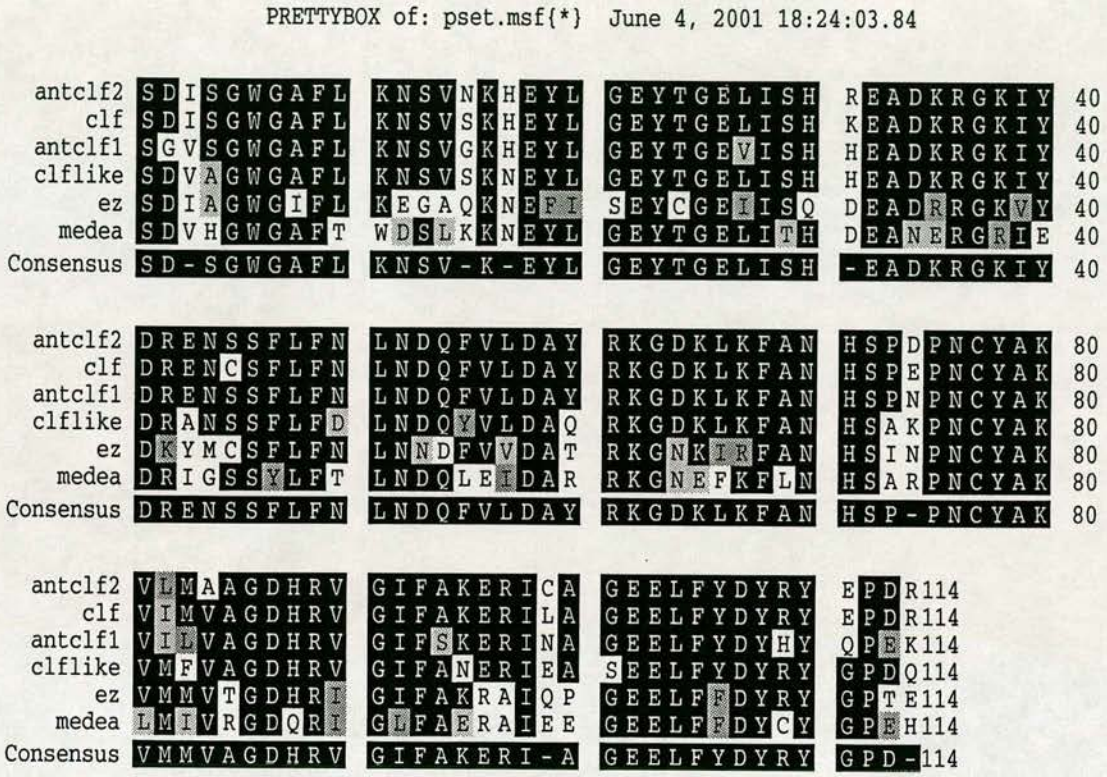


Figure 3.4 The SET domains of *ANTCLF1* and *ANTCLF2* aligned to *Drosophila Enhancer of Zeste (E(z))* and the *Arabidopsis E(z)* homologues *CLF*, *CLFLIKE* and *MEDEA*. Conserved residues in black, conservative substitutions are shaded.

3.3 RNA expression patterns of *ANTCLF1* and *ANTCLF2*

To further characterise the potential functions of these genes their expression patterns were localised by *in situ* hybridisation of DIG labelled RNA probes to tissue sections. cDNA fragments from the 5'-ends of both genes were subcloned to omit conserved sequence from the cysteine-rich and SET domains located towards the 3' end of the gene (see Chapter 2, table 2.5). These subclones were used for the *in vitro* transcription of sense and antisense RNA. The RNA was labelled by incorporation of digoxigenin-11-UTP during the transcription.

Expression of *ANTCLF1* and *2* was examined in wild-type seedlings. Longitudinal sections of young seedling were hybridised *in situ* with *ANTCLF1* and *ANTCLF2* antisense and sense probes to determine whether the genes were expressed in the shoot meristem or leaves. Young *Antirrhinum* leaf primordia emerge on either side of shoot apex from leaf initial cells (P_o). The youngest pair of leaf primordia, closest to the apex is termed P_1; older leaf primordia are numbered sequentially down the stem (P_2, P_3 etc). Successive pairs of primordia arise at 90° to one another producing a decussate phyllotaxy.

ANTCLF1 and *2* shared identical expression domains. *ANTCLF* mRNA expression was detected across the shoot meristem (Figure 3.5 a, b, d, h) and in young leaf primordia (P_1) at the periphery of the apex (Figure 3.5 a, b, c, h). Expression was also observed in more mature leaf primordia (P_3, Figure 3.5 a, c, h). Weak expression was detected in the vasculature of the stem (Figure 3.5 c, d). Sections probed with sense *ANTCLF1* and *2* revealed a low, uniform background signal (Figure 3.5 f, g). As a further control *PHANTASTICA (PHAN)* antisense probe was also hybridised to vegetative sections. The *PHAN* expression pattern has been described previously: it marks leaf initials (Po) and is also expressed in leaf primordia but is excluded from the shoot apex (Waites *et al*, 1998). A similar expression pattern was observed in these experiments (Figure 3.5e) suggesting that the rather uniform vegetative expression of *ANTCLF1* and *2* was genuine rather than a result of limitations in tissue or procedure.

The pattern of mRNA localisation of *ANTCLF1* and *ANTCLF2* in vegetative tissue was equivalent to that of *CLF* (Goodrich *et al*, 1997). *CLF* is expressed throughout the apical meristem, leaf primordia and leaves where it is thought to repress *AG*. Equivalent expression of *ANTCLF1* and *2* suggests that these genes may function during vegetative development.

The RNA expression of *ANTCLF1* and *2* during floral development was also determined. The *Antirrhinum* inflorescence meristem produces small leaf-like bracts in a spiral arrangement; floral meristems are initiated in the axils of these bracts. As a

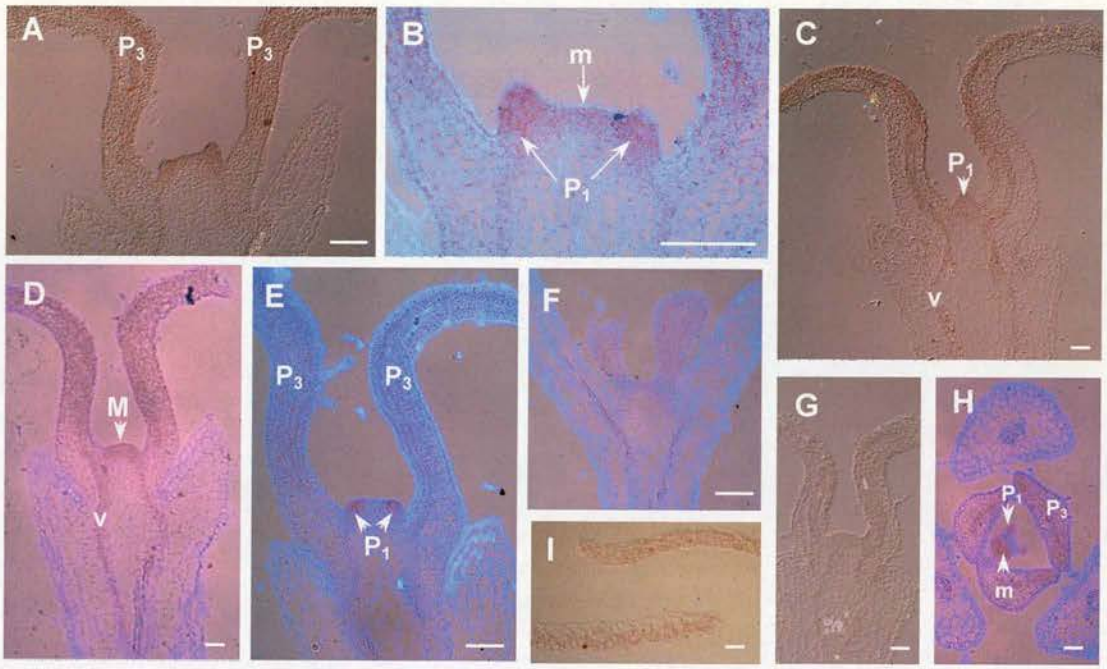


Figure 3.5 *In situ* hybridisation of *ANTCLF1* and *ANTCLF2* to longitudinal (A, B, C, D, E, F, G, I) and transverse (H) sections of vegetative apices. In B, D, E, F and H UV illumination was used under bright field, the signal is purple/brown on a background of blue epifluorescent tissue. In A, C, I and G Namarski optics were used, the signal is dark red. Young wild-type *Antirrhinum* seedlings were probed with DIG-labelled antisense *ANTCLF1* (C and I) and *ANTCLF2* (A, B, D, H) probes. *ANTCLF2* expression can be seen across the meristem (m) (B, D and H) and in young and old leaf primordia (P_1 and P_3 respectively) (A, D, H). *ANTCLF1* was expressed in similar domains, expression in young leaf primordia (P_1, C), larger leaf primordia (P_3, C) and leaves (I) is shown. *ANTCLF1* and *ANTCLF2* were also expressed in the vasculature (v) (C and D). No expression is seen in sections probed with sense *ANTCLF1* (G) and *ANTCLF2* (F). *PHAN* expression was used as a marker for specific expression in young leaf primordia (P_1) and exclusion from the meristem (E). Scale bars represent 100 µm.

result a longitudinal section through the inflorescence apex includes bract primordia and their associated floral meristems at various developmental stages in a developmental series so that the earliest stages were seen near the top of the inflorescence apex. Longitudinal sections of young *Antirrhinum* inflorescences were probed with labelled antisense and sense *ANTCLF1* and *2*. The expression pattern obtained in both cases was very similar to that observed for *Arabidopsis CLF*. Expression was detected in the inflorescence meristem and floral meristems at a number of developmental stages (Figure 3.6 b). Sections probed with sense *ANTCLF1* and *2* revealed a very low and uniform signal (Figure 3.6 a, c).

Figure 3.6d shows *ANTCLF2* expression across the inflorescence apex and also in a bract tongue. Weak expression was detected in larger bracts and in the provasulature (Figure 3.6b). Expression can be detected at the earliest stage of floral meristem development, the stage 1 eye shaped floral meristem arising at the periphery of the apex (Figure 3.6d) (developmental stages were defined according to Carpenter *et al*, 1995). Between floral meristem stages 2 to 4 a signal is present across the dome of the floral meristem concentrated in the upper cell layers (Figure 3.6b, e) and also in developing sepal primordia (Figure 3.6e). The *ANTCLF1 in situ* expression pattern mirrored that of *ANTCLF2* in the inflorescence apex and all stages of floral meristem development. However *ANTCLF1* appeared to be expressed at a slightly lower level than *ANTCLF2* and this difference was also detected in vegetative tissue. Figure 3.6f shows expression of *ANTCLF1* in an early stage 6 floral meristem, expression is visible in the developing petals, stamens and carpels, and more weakly in the sepals.

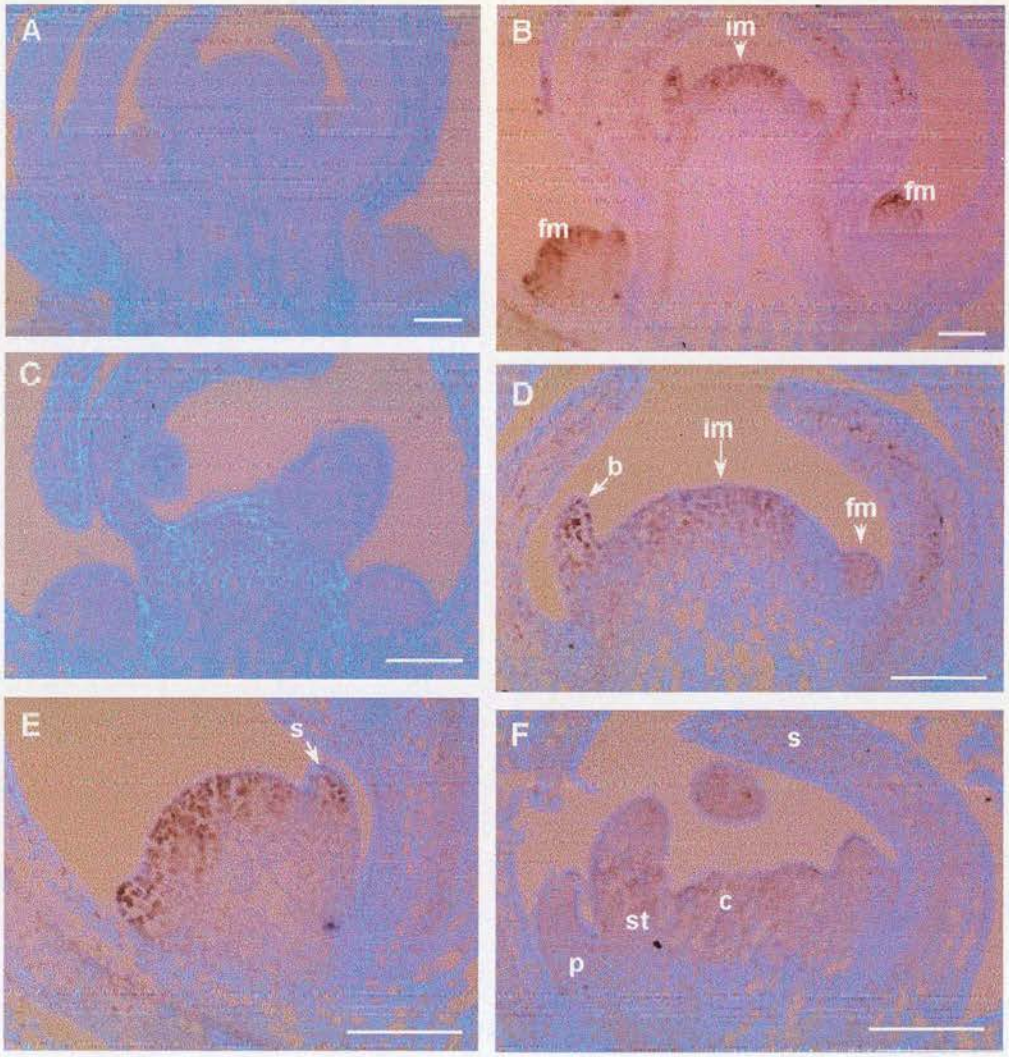


Figure 3.6 *In situ* hybridisation of *ANTCLF1* and *ANTCLF2* to longitudinal sections of wild type inflorescences. Bright field under UV illumination; the signal is purple/brown on a background of blue epifluorescent tissue. Wild type *Antirrhinum* inflorescences were hybridised with DIG-labelled antisense *ANTCLF1* (F) and *ANTCLF2* (B, D, and E) probes. *ANTCLF2* is present in the inflorescence meristem (im), young floral meristems (fm) and bract primordia (b) (B and D). A high power view of a florotypic bud (E) shows *ANTCLF2* expression across the meristematic dome and in developing sepal's (s). *ANTCLF1* was also expressed in these domains. Expression of *ANTCLF1* is shown in all four whorls of a developing flower bud (F), the carpel (c), stamen (st), petal (p) and sepal (s) whorls are shown. No expression is detected when *ANTCLF1* (A) and *ANTCLF2* (C) sense probes were used. Scale bars represent 100 µm

At late stage 6 expression of the *ANTCLF* genes is still apparent in the organ primordia in all four floral whorls. Expression of *ANTCLF1* and *ANTCLF2* then decreases in the sepal and petal whorls as they reach maturity (Figure 3.7b, d). In older flower buds expression of *ANTCLF1* and *2* becomes localised to particular tissues in the stamen and carpel whorls. Both genes are originally expressed in the central area of the gynoecium, omitting the carpel wall (Figure 3.7d) but later expression is concentrated in the developing ovules and weak or absent from the placenta (Figure 3.7c, e). Expression in the anther excludes a ring of outer connective tissue (Figure 3.7 c, d).

Since *ANTCLF1* and *2* share identical expression domains in both vegetative and floral tissue and a high sequence identity it is possible that they are functionally redundant. This implies that a double mutant of *antclf1* and *antclf2* would be required to reveal the function of these genes. Both the vegetative and floral expression domains of *ANTCLF1* and *2* mirror those of *CLF,* which was also expressed at low levels in a fairly uniform fashion. The strongest expression corresponded to areas where cells are actively dividing. The expression of *FIM-associated proteins (FAPs)* within wild-type inflorescences is very similar, occurring across the inflorescence meristem, floral meristem, in young bracts, the provasculature and in actively dividing ovule and anther tissue (Ingram *et al*, 1997). Because *ANTCLF1* and *2* are not whorl specific they are unlikely to have a direct

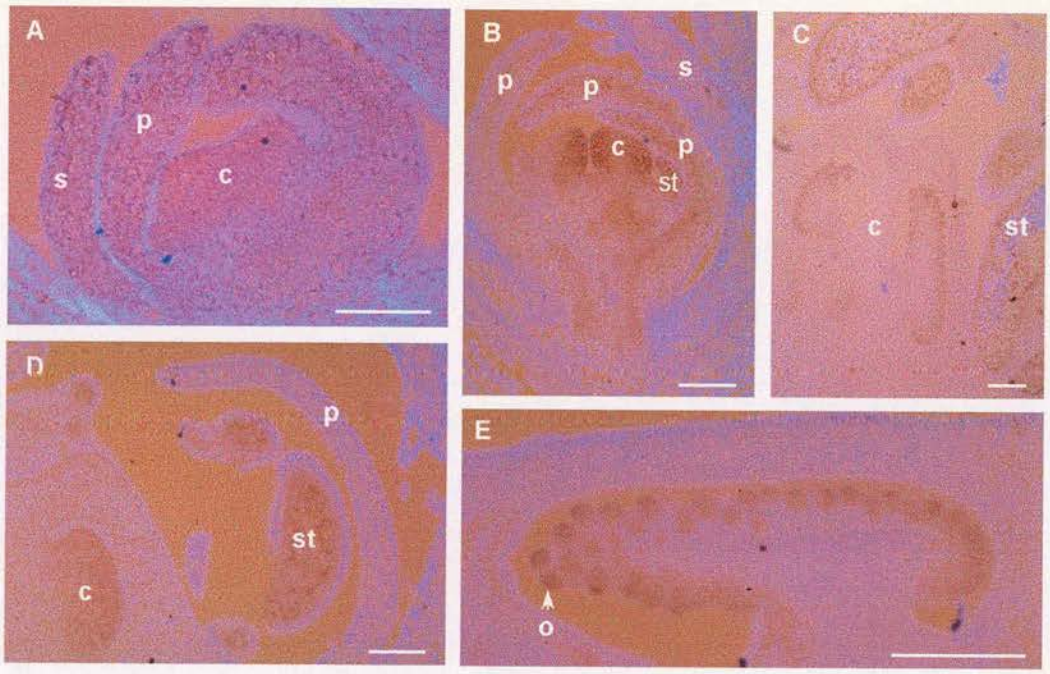


Figure 3.7 mRNA Expression of *ANTCLF1* and *ANTCLF2* in mature wild-type flower buds. Bright field under UV illumination, the signal is purple/brown on a background of blue epifluorescent tissue. Wild type *Antirrhinum* inflorescences were probed with DIG-labelled antisense *ANTCLF1* (A and D) and *ANTCLF2* (B,C and E). *ANTCLF1* is expressed in all four whorls of a stage 6 floral bud (A) including the sepals (s) and petals (p). At later stages expression declines in the sepals and petals (B (*ANTCLF2)* and D (*ANTCLF1)*) but is still visible in the stamen (st) and carpel (c) whorls. Within the carpel the *ANTCLF* genes are expressed in the region of the placenta and developing ovules but absent from the carpel wall (*ANTCLF1*, D), later expression is localised in the ovules (o) (*ANTCLF2*, C and E). Scale bar represents 100μm.

role in pattern specification, and perhaps interact with regionally specific factors. Since *ANTCLF1* and *2* are expressed during the early stages of floral development when the patterns of *abc* function genes are initiated they could be recruited to repress specific targets by localised factors.

3.4 The isolation of *antclf1* and *antclf2* mutants

3.4.1 Introduction

To determine the biological function of *ANTCLF1* and *2*, loss of function mutations were required. Since *ANTCLF1* and *2* are probably partially or completely functionally redundant, disruption of both genes may be necessary to reveal mutant phenotype and biological function. Phenotypic characterisation of a Pc-G mutant in *Antirrhinum,* a distant relative of *Arabidopsis,* would allow comparison of the function of these important developmental regulators in the plant kingdom. The floral phenotype of *antclf1* and/or *antclf2* mutants would be instrumental in determining whether the genes controlling dorso-ventrality asymmetry of the *Antirrhinum* flower interact with Pc-G genes.

Several classical mutants have been described which show altered *c* function expression, and represented candidates for *antclf* mutants. Southern blot analysis was used to determine whether these candidate mutants resulted from disruptions in *ANTCLF1* and/or *ANTCLF2* caused by transposon insertions. In some cases linkage analysis was performed to identify whether smaller alterations in *ANTCLF1* and/or *2* had resulted in the production of these mutants. As a second approach transposon mutagenised populations were screened for insertions disrupting *ANTCLF1* and *2*. This reverse genetic approach was favoured as it could detect mutations that caused

either a subtle mutant phenotype or no mutant phenotype, a situation that was likely due to the probable functional redundancy of the genes. Lastly viral-induced gene silencing was attempted, to reduce or eliminate expression of the two genes.

3.4.2 Characterisation of mutants which confer altered *c* function expression

3.4.2.1 *heptandra* (*hep*) genetic analysis

The *heptandra* (*hep*) mutation was identified in a transposon mutagenesis experiment by A. Hudson. The wild-type progenitor is not known for certain but is likely to be JI 75 which was one of the progenitor stocks for the mutagenesis experiment. The petals lobes of *hep* flowers are reduced, and stamenoid tissue resembling anthers is found on the lower petal lobes (McSteen, 1997) (Figure 3.8 a, b). This phenotype suggested that *PLE* was ectopically expressed in the second whorl and this was confirmed by *in situ* hybridisation experiments (McSteen, 1997) which showed ectopic *PLE* expression in the 2nd whorl primordia of stage 6 *hep* flowers. The *clf* mutant phenotype is also characterised by petal stamenoidy in the second whorl resulting from ectopic *c* function expression. *HEP* was therefore a strong candidate to represent an *antclf1* and/or *antclf2* mutant.

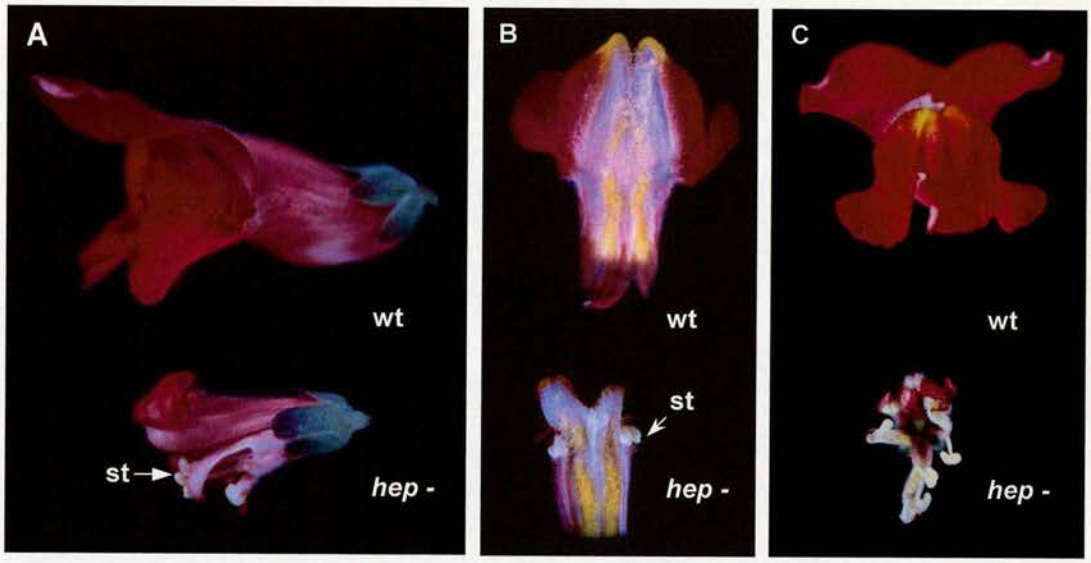


Figure 3.8 The *heptandra* (*hep*) floral phenotype. The flowers of the *hep* mutant have reduced petal lobes, which show stamenoidy (st) (A). (B) Close up to show anther sacs (st) on either side of the lower petal lobe in the hinge region. In severe cases the petal lobes are absent and the corolla tube split so that the third whorl stamens protrude (C).

Two F2 families (A11 and A25) were grown (gift of A. Hudson), each derived from crosses between *hep* (likely background JI 75) and wild-type JI 98. Phenotypic scoring of the two families revealed a range of phenotypes similar to those described by McSteen (1997) in F2 families from an outcross to Sippe 50 (wild-type) (Table 3.1). The weakest floral phenotype consisted of petals with lateral incisions or 'nicks' in the middle of the upper and lower petal lobes. A weak hep floral phenotype was observed in which the petal lobes were reduced but stamenoidy was rare, or only occurred late in development on lateral shoots. Strong hep floral phenotypes were characterised by stamenoidy of the lower petal lobes with two anther sacs present on either side of the lateral petal towards the hinge region (Figure 3.8b). In one family (A25) two plants were observed which had a few flowers with a severe phenotype (Figure 3.8c) in which the petal lobes were absent and the corolla tube severely reduced in length. The corolla tube was split and the 3rd whorl stamens extended beyond the shortened petals. Plants displaying the strong floral hep phenotype were often bushy because several of the lower flowers were transformed into lateral shoots, the occasional transformation of a flower into an inflorescence shoot was also noticed by McSteen (1997).

In F2 populations from crosses to the Sippe 50 genetic background, McSteen (1997) observed that the hep mutant phenotype did not segregate in a 3:1 ratio characteristic of a single recessive mutant. Several of the populations however showed a high

probability of the 3:1 ratio with a chi-squared test if the nicked phenotype was assumed to be unrelated to *hep*. This is possible since the nicked phenotype has also been observed in other crosses (McSteen, 1997). As an alternative McSteen (1997) suggested that *hep* might be a double mutant, in this case the strong hep phenotypes would be classed as double mutants whereas nicked and weak hep would represent the single mutant.

Here, chi-squared analysis was used to analyse the two F2 families (A11 and A25) segregating for *hep*, to determine the probability that *hep* was a single recessive mutant. If the nicked phenotype is assumed to be unrelated to *hep* then A11 segregates 24 Hep^{+}: 5 *hep* and A25 segregate 33 Hep^{+}: 9 *hep*. Chi-squared analysis reveals high probability values for a 3:1 segregation in both families (A11 ($P>0.1$) and A25 ($P>0.5$)).

In contrast the segregation of floral phenotypes for A11 and A25 does not suggest that *hep* is a double mutant, in this case a 9:6:1 ratio would be expected, the strong hep phenotype representing the double mutant. Neither line approaches this ratio whether nicked is regarded as wild-type or single mutant. The most straightforward hypothesis to account for the data is that *hep* is a single recessive mutant with variable penetrance, giving rise to a range of intermediate to severe hep phenotypes. However a larger sample size would be needed to confirm this.

To determine whether the nicked phenotype is unrelated to *hep* A25 plants with a weak hep floral phenotype were crossed to A24 (a homozygous *hep* line), to confirm that these were homozygous *hep* rather than single mutants from a *hep* double mutant. In addition both nicked and weak hep plants from A11 and A25 were selfed. Future analysis of the phenotypes resulting from these crosses should be able to confirm that *hep* is a single recessive mutant.

	Family	Family
Floral Phenotype	A11	A25
Wild-type	21	19
Nicked	3	14
Weak Hep	3	4
Strong Hep	2	3
Severe Hep	0	2
Total	29	42

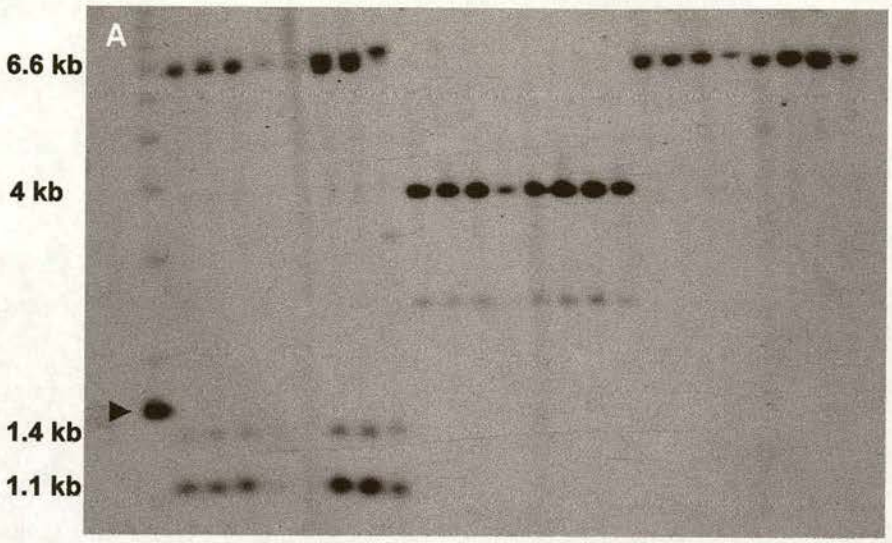
Table 3.1 Floral phenotypes of two families segregating for *hep*, A11 and A25

3.4.2.2 Molecular analysis of *HEPTANDRA*

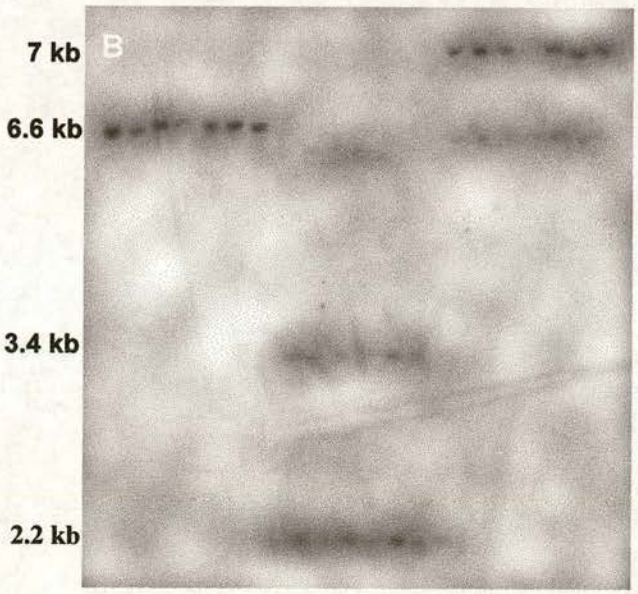
Genomic DNA was extracted from plants of the segregating F2 family A11 and subjected to Southern blot analysis to determine whether the *hep* mutant resulted from a transposon insertion in *ANTCLF1* and/or *ANTCLF2*. Genomic DNA was extracted from three plants with a weak hep floral phenotype, two plants with a strong hep floral phenotype and one HEP^{+} wild-type plant. Genomic DNA was also

prepared from the parental lines JI 75 and JI 98. The genomic DNA was digested with three different restriction enzymes *Eco* RI, *Bgl* II and *Eco* RV, separated by agarose gel electrophoresis and blotted onto Nylon membranes. Two blots were prepared which were hybridised at high stringency with molecular probes produced from cDNA fragments of *ANTCLF1* and *ANTCLF2* (Figure 3.9 a, b). The cDNA fragments routinely used to produce probes for Southern blot analysis were the *ANTCLF1* cDNA pcc2b that includes 1.7 kb of 3' sequence, and the *ANTCLF2* cDNA pcc4, a 3kb full length clone. Despite presence of the conserved SET domain and 2nd cysteine rich domain in both of these cDNA fragments the two probes produced distinct patterns when probed at high stringency to RFLP Southern blots suggesting that they were specific for *ANTCLF1* or *ANTCLF2*. No polymorphism co-segregated with the *hep* mutants when the Southern blots were hybridised with either *ANTCLF1* or *ANTCLF2*. This suggests that the *hep* mutant is not caused by a transposon insertion which disrupts *ANTCLF1* or *2,* however it does not rule out the possibility that a transposon initially inserted into either gene and then excised imprecisely thus mutating the gene, RFLP analysis would be necessary to detect these changes.

Attempts were made to detect RFLPs between various wild-type lines for *ANTCLF1* and 2 so that linkage analysis could be undertaken. Genomic DNA from five wild-type lines (Sippe 50; JI 98; JI 75; JI 69; T704) was digested with a variety of restriction enzymes and hybridised on Southern blots with an *ANTCLF2* probe by N. Ehrenberg. A couple of RFLP's were detected between the Sippe 50 and the other



M = 1 kb ladder; 1 and 2 = strong *hep*; 3 to 5 = weak *hep* ; 6 = *HEP+* ; 7= JI75; 8 = JI98



1 = strong *hep*; 2 to 4 weak *hep* ; 5 = *HEP+* ; 6 = JI98; 7 = JI75

Figure 3.9 Southern analysis of *heptandra* with an *ANTCLF1* (A) and *ANTCLF2* (B) probe. An arrow indicates a marker band of 1.6 kb.

wild-type lines, however no RFLP was detected between JI 75 and JI 98. The blots were stripped and hybridised with an *ANTCLF1* probe, Again a few RFLPs were detected between Sippe 50 and the other wild-type lines, but none were detected between JI 75 and JI98. This meant that RFLP analysis could not be performed to determine linkage between *hep* and the *ANTCLF* genes, in the F2 from the JI 98 cross.

3.4.2.3 Molecular analysis of *polypetala (poly)*

The semi-dominant *polypetala (poly)* mutant arose spontaneously in a cross between JI 69 and JI 75. Heterozygous plants usually produce one extra whorls of petals giving a whorl order sepal, petal, petal, stamen, carpel (Figure 3.10a). Homozygous plants produce a more extreme phenotype. The flowers have a first whorl of sepals and an indeterminate number of petals. No stamens or carpels are produced in the centre of the flower (McSteen, 1997; McSteen, 1998) (Figure 3.10a). This phenotype results from a reduction in *PLE* in the floral meristem (McSteen, 1998). Because *poly* is semi dominant, it could be a gain of function mutation, and if so the wild-type function of the gene would be the down regulation of the *c* function gene PLE in the outer floral whorls. The *Arabidopsis CLF* gene also represses a *c* function gene in the outer whorls making *poly* a candidate to represent *antclf1* and/or *antclf2* mutants.

An F2 family was grown derived from a cross between *poly* (JI 69/JI 75) and the wild-type Sippe 50 (gift of R. Carpenter). Genomic DNA was extracted from plants that were homozygous *poly*, heterozygous *poly* and *POLY*$^{+}$. Genomic DNA was analysed by Southern blotting together with the parental lines JI 69, JI 75 and Sippe

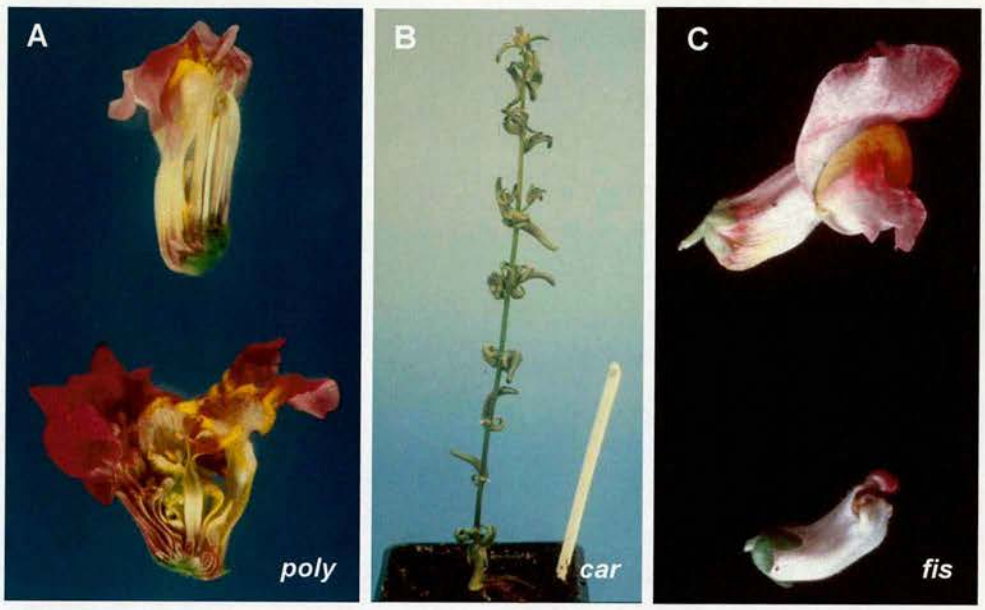


Figure 3.10 The *polypetala* (*poly*) (A), *carnosa* (*car*) (B) and *fistulata* (*fis*) (C) mutants. Heterozygous *poly* flowers produce an extra whorl of petals (top, A) whereas homozygous *poly* flowers produce an indeterminate number of petals internal to the sepal whorl (bottom, A). Mutants of *car* are dwarfed and have small rolled leaves (B). The petal lobes of the *fis* mutant flower are severely reduced (bottom, C) relative to wild-type flowers (top, C).

50. Restriction Fragment Length Polymorphism's had previously been detected in *Dra* I and *Hinc* II digests between Sippe 50 and JI 75/JI 69 when an *ANTCLF2* probe was used. An *ANTCLF2* probe was hybridised on a Southern blot containing genomic DNA from the *poly* population that had been digested with *Hind* III, *Hinc* II and *Dra* I (Figure 3.11b). No novel fragments co-segregated with the *poly* mutation in any of the digests indicating that *poly* is not caused by a transposon insertion in *ANTCLF2*. RFLP analysis was used to test for co-segregation of *ANTCLF2* with the *poly* mutation. Fragments of 6.6 kb and 5.1 kb are present in *Hinc* II digests in the Sippe 50 background (Lane 2) but not JI 69 or JI 75 (Lanes 1 and 3). These Sippe 50 fragments do not co-segregate with the *poly* mutant, for example a homozygous *poly* sample (Lanes 7) has the wild-type Sippe 50 bands (6.6 kb and 5.1 kb). This result is confirmed by observation of the *Dra* I digest. Sippe 50 (Lane 2) produces a 2.9 kb band, not found in digestions of JI 69 or JI 75 genomic DNA (Lanes 1 and 3). In addition, JI 69/JI 75 produce a 3.2 kb band, absent from the Sippe 50 lane. If co-segregation was present, the homozygous *poly* sample (Lane 7) would not have the Sippe 50 band (2.9 kb) and the heterozygous *poly* sample (Lane 5) would have the JI 69/75 band (3.2 kb).

An *ANTCLF1* probe was also hybridised on a Southern blot containing genomic DNA from the *poly* population that had been digested with *Hind* III, *Hinc* II and *Dra* I (Figure 3.11a). Again no novel fragments co-segregated with the *poly* mutation, indicating that *poly* does not result from a transposon insertion which disrupts *ANTCLF1*.

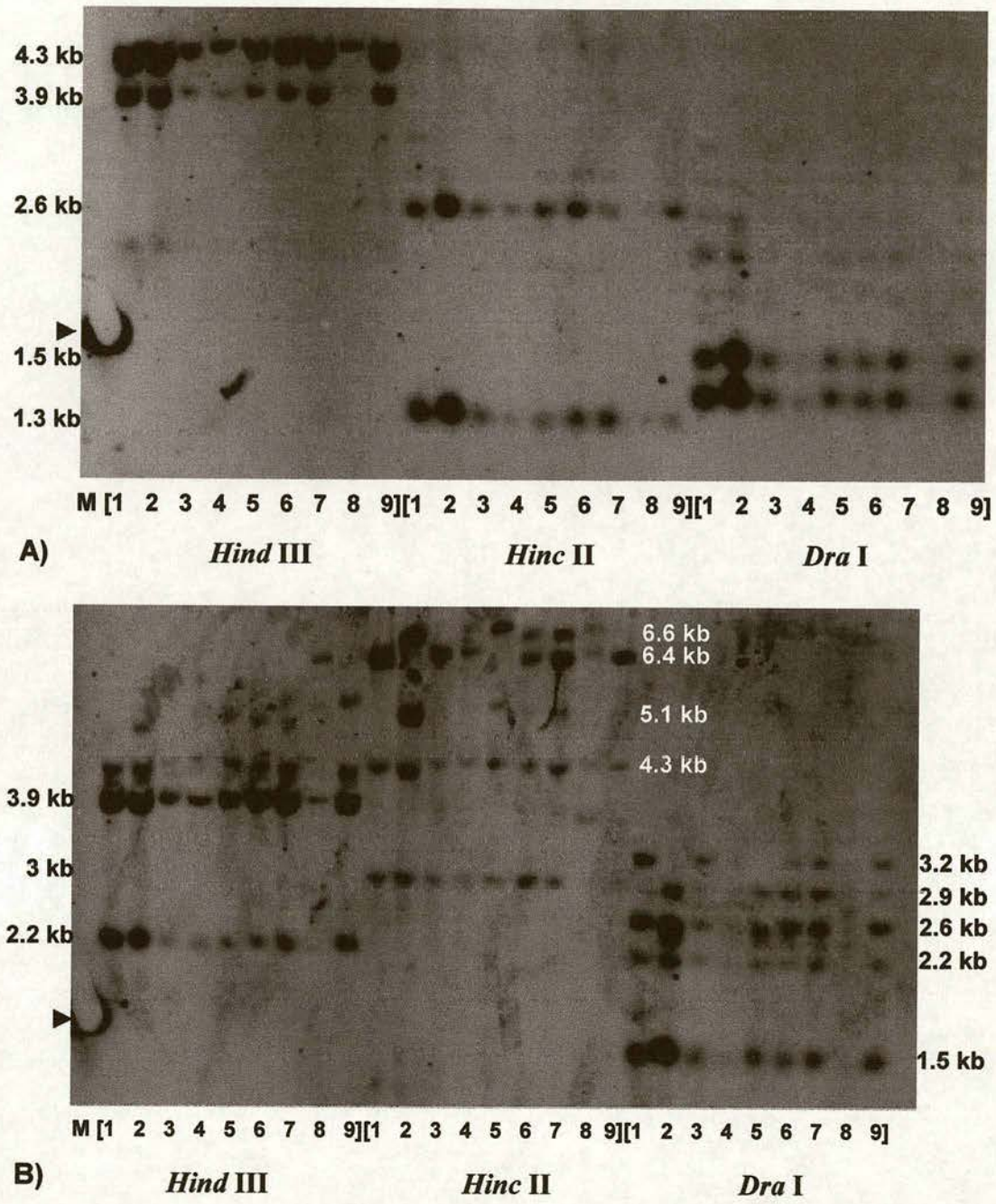


Figure 3.11 Southern analysis of *polypetala (poly)* with an *ANTCLF1* (A) and *ANTCLF2* (B) probe. (M = 1 kb marker; 1 = JI 69 (wt); 2 = sippe 50 (wt); 3 = JI 75 (wt); 4 = *POLY+*; 5 and 6 = *poly-/POLY+* (het); 7 to 9 = *poly-/poly-*). Arrow indicates the 1.6 kb marker band.

3.4.2.4 Molecular analysis of *fistulata (fis)* and *carnosa (car)*

The recessive mutant *fistulata (fis)* originates from the Gatersleben collection (Stubbe, 1966). The petals of *fis* have reduced lobes and stamenoidy resulting from ectopic expression of *PLE* in the second whorl (McSteen, 1997; McSteen, 1998, Motte, 1998) (Figure 3.10c). The classical dominant mutant *carnosa (car)* is also from the Gatersleben collection. The mutant is strongly retarded in growth and has small curled leaves and few flowers (Figure 3.10b).

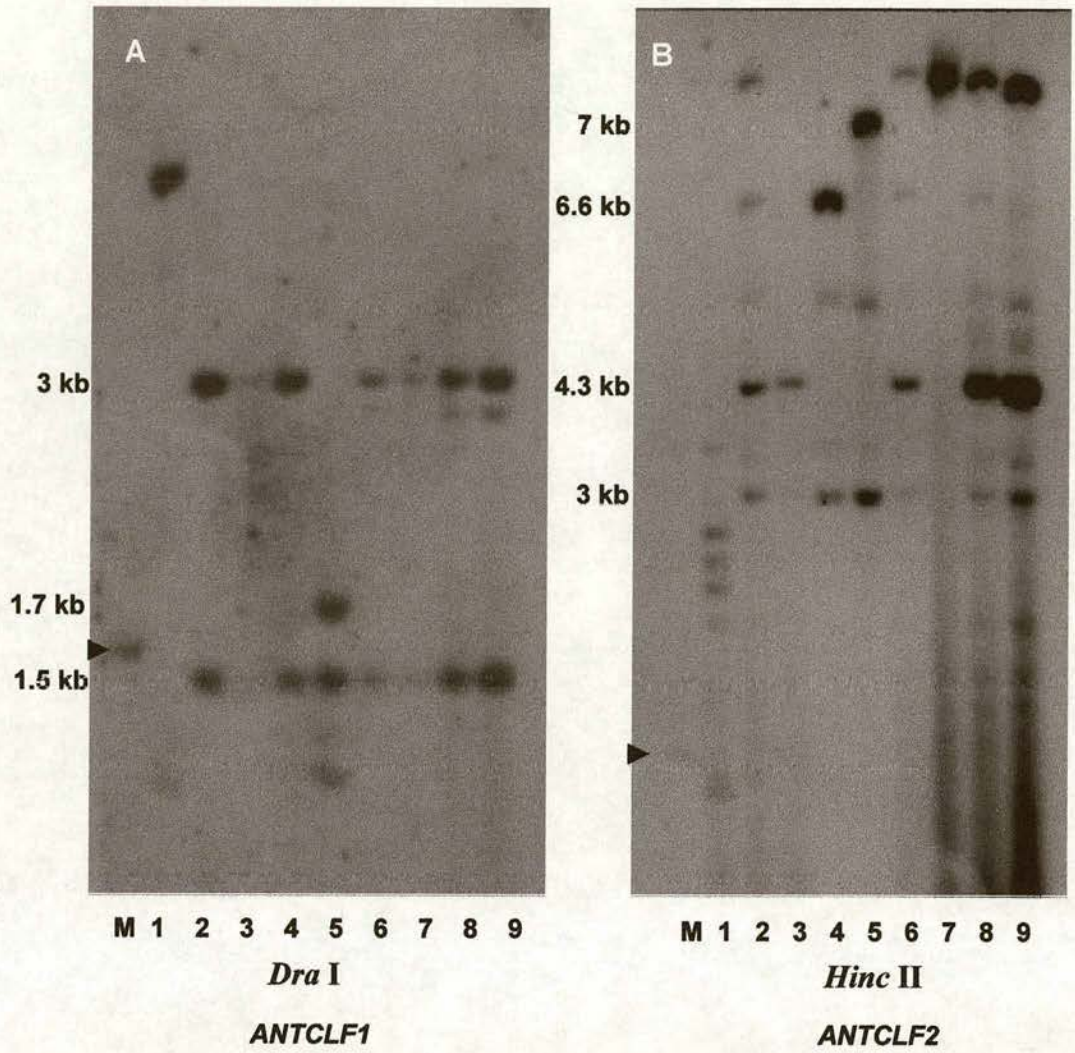
Southern blot analysis was used to determine whether *fis* or *car* resulted from disruptions in either *ANTCLF1* or *ANTCLF2*. Genomic DNA was extracted from three homozygous *fis* lines, derived from an F2 cross *fis*- (Sippe 50) x FIS^+ (JI 75). Genomic DNA was also extracted from an F2 cross (*car*- (Sippe 50) x CAR^+ (JI 98); from Z. Schwarz-Sommer) by N. Ehrenberg, one *car*- bulked sample was analysed along with a CAR^+ bulked sample and two single CAR^+ samples.

The enzyme *Dra* I had previously been shown to produce an RFLP between the wild-type lines Sippe 50 and JI 98 on blots hybridised with the *ANTCLF1* probe pcc2b. Therefore this enzyme was used on a blot hybridised with *ANTCLF1*. Although JI 75 rather than JI 98 was used in the *fis* F2 cross, both of these wild-type lines had produced the same band sizes after *Dra* I digestion on previous RFLP blots. These lines share similar band sizes in many digests because JI 98 was derived from

a cross between JI 75 and JI 69. *Hinc* II was used to digest genomic DNA from *fis* and *car* lines on a blot hybridised with *ANTCLF2* because an RFLP between JI 98 and Sippe 50 had previously been detected on RFLP blots.

No large band shifts co-segregated with the *car* mutant on blots hybridised with *ANTCLF1* or *2*. This indicates that the *car* mutant does not result from transposon insertion in the *ANTCLF* genes. Strangely in the *ANTCLF1* hybridised blot there is no evidence that the predicted RFLP is segregating. This may indicate that the sample used had not been from a JI 98 cross. Because of this linkage analysis was not possible.

Problems also arose in *fis* analysis: 1) One *fis* sample (Figure 3.12a; Lane 1) produced a band of an unexpected large size after *Dra* I digestion and hybridisation with *ANTCLF1,* which may have indicated transposon insertion, however an additional two *fis* samples (Figure 3.12a; Lanes 2 and 3) did not produce this band ruling out this possibility, the same sample also produced unusual bands after digestion with *Hinc* II and hybridisation with *ANTCLF2* (Figure 3.12b; Lane 1); 2) the sample size was too small and did not include *fis* segregants precluding RFLP analysis. To summarise the analysis of *fis* was insufficient to determine whether *fis* results from a transposon insertion in *ANTCLF1* or *2*, linkage analysis was also unsuccessful. A good RFLP has been detected between JI 75 and JI 98 and Sippe 50 in *Sca* I digests when an *ANTCLF1* probe is used, this could be used together with



M = 1kb ladder; 1 to 3 = *fis* ; 4 = sippe 50 (wt); 5 = JI98 (wt); 6 = *car* ; 7 to 9 = *CAR+*

Figure 3.12 Southern analysis of *fistulata (fis)* and *carnosa (car)* with an *ANTCLF1* and *ANTCLF2* probe. Arrow indicates the 1.6kb marker band.

previously confirmed *ANTCLF2* RFLPs to carry out linkage analysis of *fis* to *ANTCLF1* or 2.

3.4.3 Reverse genetic screens for transposon insertions disrupting *ANTCLF1* and *ANTCLF2*

PCR screening of transposon mutagenised lines has been used for reverse genetic screens, to identify insertions that disrupt a gene of interest (e.g Davies *et al*, 1999). Because PCR amplification is extremely sensitive, it can detect a very rare template, for example DNA from a single mutant plant in a mixture of DNA from several thousand plants.

Similarity in both sequence and expression suggests that *ANTCLF1* and 2 are functionally redundant thus an *antclf1* or *antclf2* mutant may have no distinguishable phenotype. PCR screening is particularly useful in these cases as single plants with a transposon insertion in the gene of interest are identified not by phenotype but at a molecular level.

The technique involves screening pools of genomic DNA extracted from large populations of *Antirrhinum* plants, which have undergone a transposon mutagenesis. The insertion of a transposon in a gene of interest is identified by the detection of a gene specific PCR band produced from a gene specific and transposon primer. The

superpool in which the candidate band was detected is then deconvoluted so that eventually the insertion can be traced to a single plant.

Two large screens were used to search for insertions that disrupt *ANTCLF1* and *ANTCLF2* . The John Innes *Antirrhinum* screen (9 super pools each produced from between 8 and 9 '450' pools; obtained from E. Keck) and the Gatersleben screen (MAM pools 1-20 and A to V; also from E. Keck). The Gatersleben screen includes morphological mutants from the Gatersleben collection. Gene specific primers were designed from the cDNA of *ANTCLF1* and *ANTCLF2* (Figure 3.13). The primers, which were designed from cDNA sequence, were made compatible to amplify genomic DNA *i.e.* intron-exon boundaries were avoided. Partial genomic sequence from *ANTCLF1* had showed that 4 intron-exon boundaries are shared with *CLF*, suggesting that the intron-exon structure of *CLF* and the *ANTCLF* genes has been well conserved therefore *CLF* intron-exon boundaries were used to indicate the intron-exon boundaries of the *ANTCLF* genes. To test whether the gene specific primers were capable of amplifying genomic DNA, PCR reactions were performed using pairs of gene specific primers to amplify *Antirrhinum* genomic DNA (an example is shown on Figure 3.14a; C1 and C2). Gene specific primers were not designed at the 5' end of *ANTCLF1* since 5' RACE had not yet been carried out to identify this portion of the gene. Each gene specific primer was used in a PCR reaction with one of three different transposon primers TAM3 (right border), TAM3 (left border) and CACTA (designed from the conserved subterminal region of TAM 1, 2, 4 and 5). DIG probes used in the Southern blotting of PCR screening gels were

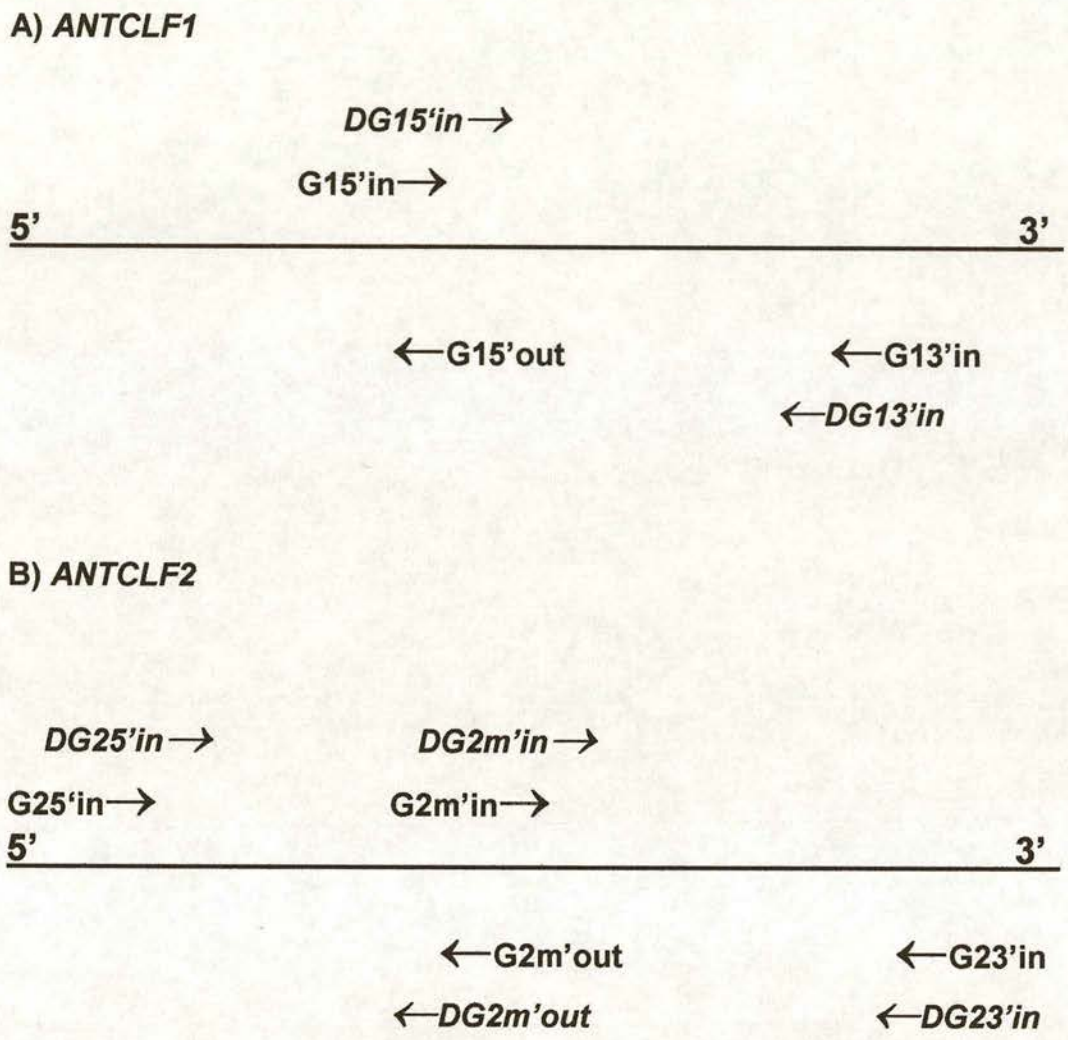


Figure 3.13 Schematic diagrams to show the gene specific *ANTCLF1* (A) and *ANTCLF2* (B) primers used in PCR screening of transposon mutagenised lines. Gene specific primers are indicated as G25'in, G2m'out etc whereas primers used to produce digoxigenin probes for southern blot analysis of PCR screens are indicated in italics e.g. *DG2m'out.*

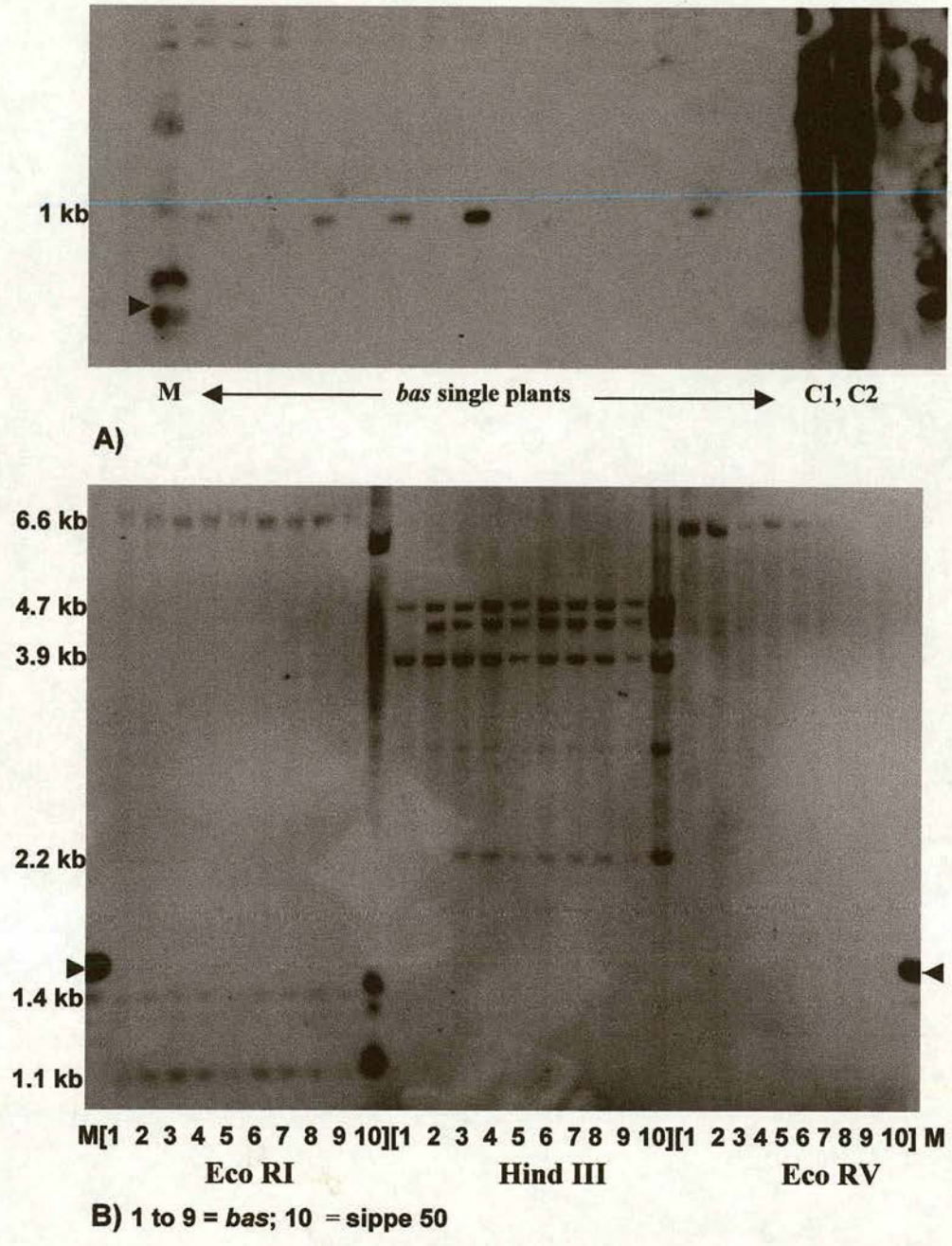


Figure 3.14 PCR and southern blot analysis of *basilutea*. 40 plants from a segregating population of *basilutea* were grown. The PCR screen was carried out using the gene specific primer G2min (*ANTCLF2)* and a CACTA internal transposon primer, figure (A) shows a southern blot of a PCR reaction from 16 of the plants, probed with *ANTCLF2*. Genomic controls C1 (*bas* genomic DNA) and C2 (*elongata* genomic DNA) show that when two gene specific primers are used (G2min and G23'in) a strong band hybridises. (B) Southern analysis of *bas* single plants using an *ANTCLF2* probe. Arrows show the 1.6 kb marker band, M = 1 kb marker.

designed internal to the gene specific probes to prevent cross hybridisation to primer sequences.

Several candidate bands were identified in superpools from the John Innes screen, but in each case the bands were not found in PCR amplification of constituent sub-pools suggesting that they were artefacts. It is possible that transposon insertion had occurred, but only in a somatic sector, making it undetectable in the constituent subpools. The one candidate band was discovered using the Gatersleben screen. The ~1kb band was identified from Southern blotting of a PCR reaction using the gene specific primer G2min (*ANTCLF2)* and the CACTA transposon primer. The band was identified in pools J and 10 which corresponded to MAM 33 (Basilutea) on the grid. Unfortunately this band could not be separated from a large transposon generated band of a similar size therefore the band could not be isolated and sequenced. To isolate the candidate band, nested PCR was attempted using three primers were used that were internal to the gene specific primer (G2min) and one primer external to CACTA. This was mainly unsuccessful, although one band was obtained with the first nesting reactions. This band was isolated and sequenced but did not correspond to *ANTCLF2*. It was therefore not possible to determine whether the Gatersleben candidate band was caused by a genuine transposon insertion in *ANTCLF2*.

Genomic DNA from 40 *basilutea* plants was tested using the original PCR screening reaction, bands of a similar sizes (~ 1000 bp) were identified in approximately one

third of the plants (Figure 3.14a). To confirm whether *basilutea* was produced from a transposon insertion in *antclf2,* Southern analysis was performed using five *bas* samples which had produced the candidate band and 4 that had not. No large band shift co-segregated with the candidate band samples to suggest transposon insertion. In conclusion the band was found to be an artefact.

3.4.4 Viral Induced gene silencing

Infection of *Nicotiana benthamiana* with viral-vectors carrying sequence from an endogenous plant gene has been shown to initiate viral induced gene silencing (VIGS) of the inserted gene and the endogenous gene (Ruiz *et al*, 1998). VIGS involves an RNA-mediated plant response against the virus believed to be initiated by the presence of short double-stranded RNA molecules in infected cells, followed by the systemic spread of a silencing signal that directs sequence specific RNA degradation targeted against viral sequences (Hamilton and Baulcombe, 1999; reviewed in Baulcombe, 1999). For example, when potato virus X (PVX) vectors carrying coding sequences from the phytoene desaturase (PDS) gene were used to infect *Nicotiana benthamiana*, the upper leaves became bleached indicating silencing of the endogenous PDS mRNA (the PDS protein usually protects against photobleaching) (Ruiz *et al*, 1998).

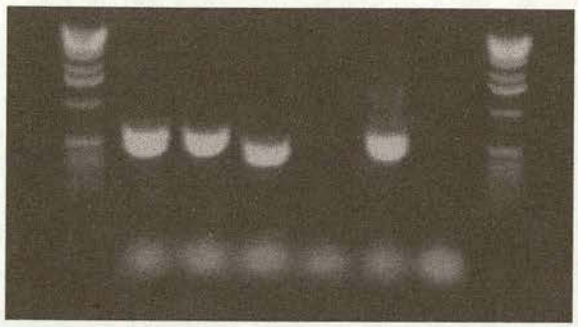
Here, VIGS was employed in an attempt to silence *ANTCLF1* and/or *2* in *Antirrhinum*. The experiments were carried out in collaboration with S. MacFarlane of the Scottish Crop Research Institute, Dundee. Gene sequence from *ANTCLF1* and *ANTCLF2* was inserted into the pK_2O.GFPc viral plasmid (gift of S. MacFarlane). This pUC-based plasmid contains the sequence encoding the RNA2 particle of the Tobravirus, Tobacco rattle virus (TRV). RNA2 is not essential for infectivity so that sequences from a gene of interest can be inserted into it without an effect on infectivity. TRV has another RNA particle, RNA1, which is essential for infectivity (MacFarlane and Popovich, 2000). TRV was chosen because it is able to infect meristematic regions and because the literature shows that TRV has a wide host range. TRV produces a systemic infection in tomato, petunia and *Arabidopsis* (MacFarlane and Popovich, 2000) suggesting that it may also be able to infect *Antirrhinum*. *Antirrhinum* species have not previously been used in VIGS experiments.

The GFP element of the pK_2O.GFPc plasmid was removed and replaced by three separate *ANTCLF* inserts. One non-conserved insert was produced from both *ANTCLF1* and *2*. These non-conserved inserts contained sequence found between the 1^{st} and 2^{nd} cysteine rich domains. Insertion into pK_2O produced pK_2O.G1nc and pK2O.G2nc. The third insert contained highly conserved sequence from *ANTCLF1* which included sequence encoding the nuclear localisation domain and the 2^{nd} cysteine rich domain. VIGS has been shown to target any endogenous gene with

>80% sequence similarity to the virally targeted sequence (Ruis *et al*, 1998). The conserved *ANTCLF* insert (400 bp) shared 86.4% sequence identity to the same region in *ANTCLF2*, making it likely that a VIGS event would silence both *ANTCLF* genes, to produce an effective "double mutant" phenotype.

The completed vector constructs $pK_2O.G1nc$, $pK_2O.G2nc$ and $pK_2O.G1c$ were transcribed and used to infect *N. benthamiana* in a mixed infection together with infective TRV RNA1 by S. MacFarlane. Infective plant material produced from this infection was used as inoculum to infect *Antirrhinum*.

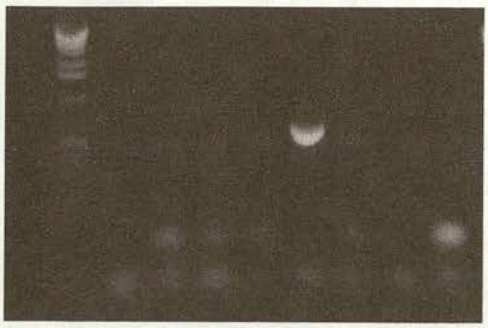
Approximately ten young (7-9 leaves) JI 75 *Antirrhinum* plants were infected with the $pK_2O.G1nc$ containing inoculum, and the same number with the $pK_2O.G2nc$ and $pK_2O.G1c$ containing inoculums. Infection was monitored by RT-PCR using primers designed to amplify TRV RNA particles 1 and 2. S. MacFarlane detected RNA1, and RNA2 (of the correct size considering the addition of the *ANTCLF* inserts) at six days post inoculation (dpi) in leaves inoculated with $pK_2O.G1nc$, $pK_2O.G2nc$ and $pK_2O.G1c$. Systemic spread of the virus was followed by detection of viral RNA1 and RNA2 in leaves that had not been inoculated, but were close to the site of infection. At 6 dpi RNA1 and RNA2 were not detected in these leaves, however at 11 dpi RNA2 was detected in both originally inoculated leaves and in a non-inoculated leaf from a plant infected with the $pK_2O.G1c$ construct (Figure 3.15a). S. MacFarlane observed a similar result at 15 dpi confirming low systemic spread of the RNA2 from this $pK_2O.G1c$ infected plant (results not shown). To test whether the



M 1 2 3 4 5 6 M

A) Viral infection at 11 days after infection (dpi)

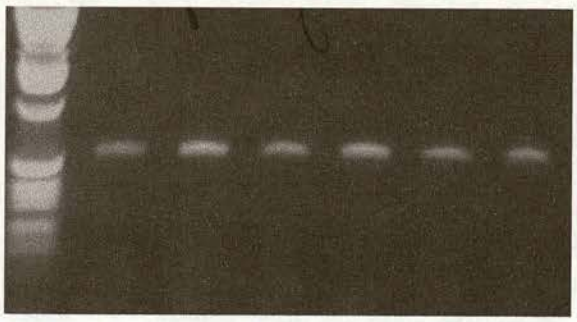
1, 3 and 5 are leaves inoculated with $pK_2O.G1c$, $pK_2O.G1nc$ and $pK_2O.G2nc$ respectively. 2, 4 and 6 are non-inoculated leaves from plants infected with $pK_2O.G1c$, $pK_2O.G1nc$ and $pK_2O.G2nc$ respectively



M 1 2 3 4 5 6 7 8

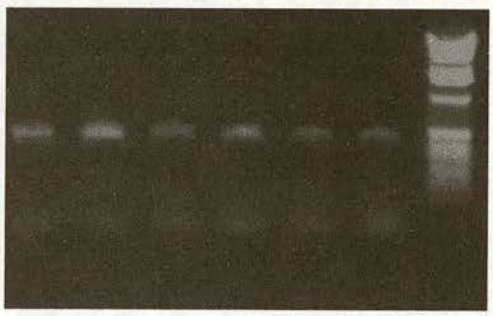
B) Systemic infection at 20 days dpi

1 and 2 are non-inoculated leaves from a plant infected with $pK_2O.G2nc$. 3, 4, 5 and 6 are non-inoculated leaves from two separate $pK_2O.G1c$ infected plants. 7 and 8 are control samples.



M 1 2 3 4 5 6

C) Expression of *ANTCLF1* in infected plants



1 2 3 4 5 6 M

D) Expression of *ANTCLF2* in infected plants

1 and 3 are lower leaf samples from two separate $pK_2O.G1c$ infected plants. 2 and 4 are upper leaf samples from the same $pK_2O.G1c$ infected plants. 5 is a control non-infected plant and 6 is a mock-inoculated sample

Figure 3.15 Transgenic Tobravirus infection of *Antirrhinum* with the constructs $pK_2O.G1nc$, $pK_2O.G2nc$ and $pK_2O.G1c$. A) and B) RT-PCR was used to detect the viral particle RNA2 of TRV, primers were designed from RNA2 sequence by S. MacFarlane. A) Shows that at 11 dpi transgenic RNA2 from all three constructs has infected within inoculated leaves. Systemic spread of infection is indicated by the presence of RNA2 in a non-inoculated leaf infected with $pK_2O.G1c$ (lane 2). B) At 20 dpi RNA2 is present in one sample from a non-inoculated leaf from a separate $pK_2O.G1c$-infected plant (lane 5). C) and D) gene specific primers designed from *ANTCLF1* and *2* (no. 44-47 table 2.2) were used in RT-PCR reactions to detect whether expression of endogenous *ANTCLF1* or *2* had decreased as a result of a VIGS event in $pK_2O.G1c$ infected plants (at 20 dpi), no decrease was detected.

pK_2O.G1c RNA2 was spreading systemically in other infected plants further RT-PCR experiments were carried out at 20 dpi (Figure 3.15b). These experiments detected the RNA2 in a non-inoculated leaf sample from another pK_2O.G1c infected plant, reinforcing the previous results. Systemic leaf sample were also taken from younger leaves (further from the original site of infection) from the infected plant sampled at 11 dpi. No RNA2 was detected in these leaves, however, suggesting that any systemic infection occurring in the pK_2O.G1c infected plants was likely to be slow and localised close to the original site of infection. A VIGS event may/or may not require systemic spread of the virus since the VIGS process itself is believed to include the production of a systemic VIGS signal. Systemic silencing was monitored by observing any phenotypic change, particularly in the upper section of infected plants. Wild-type plants and plants mock inoculated with water and carborundum were used as controls. No distinct phenotypic change was detected, although the plants did have curled leaves due to environmental stress so that any subtle change in phenotype would have been difficult to detect. Because of this a new batch of wild-type *Antirrhinum* plants (JI 98) were infected with the three different constructs. These plants were younger than the first batch of JI 75 plants, only having five to six leaves at the time of inoculation. Three to four of the upper leaves were inoculated and the phenotype of these plants was monitored regularly. A few of the infected plants produced flowers with a semi-peloric or peloric phenotype but this was believed to be due to environmental causes as these plants had been shifted from one growth room to a separate growth room, JI 98 is more susceptible to such shifts (D.Bradley personal communication).

RT-PCR using gene specific primers from *ANTCLF1* and *2* was used to determine whether or not endogenous *ANTCLF1* and/or *ANTCLF2* RNA was being silenced in pK_2O.G1c infected plants, in which systemic infection had previously been detected. The gene specific primers were designed from regions of *ANTCLF1* and *2* that had not been included in the pK_2O constructs to prevent RNA2 viral RNA being detected. At 20 dpi total RNA was extracted from an upper leaf and a lower leaf (close to the site of infection) from two different pK_2O.G1c infected plants. After reverse transcription, RT-PCR was carried out using both *ANTCLF1* and *ANTCLF2* specific primers (Figure 3.15 c and d respectively). No significant difference in the level of endogenous *ANTCLF1* or *ANTCLF2* RNA was detected when compared to control samples. This result indicated that a VIGS event had not occurred. Further experiments involving VIGS in *Antirrhinum* will need to be conducted before the technique can be used successfully in this species.

3.5 Discussion

3.5.1 *ANTIRRHINUM CLF 1* and *2* are the *Antirrhinum* homologues of the *Arabidopsis CURLY LEAF* gene.

Two homologues of the *Arabidopsis* Pc-G gene *CLF* were identified and characterised from *Antirrhinum majus* and named *ANTIRRHINUM CLF 1* and *2* (*ANTCLF1* and *2*). The proteins encoded by these *Antirrhinum* homologues shared greater homology with CLF than any other plant Pc-G protein. However ANTCLF1 and 2 were more similar to each other than either was to CLF, suggesting that the two *Antirrhinum* genes arose from a recent gene duplication. A number of domains were identified which had been highly conserved between the *ANTCLF* genes and *CLF*.

The carboxy-terminal SET domain of *Arabidopsis CLF* and other *E(z)* homologues has been well conserved in *ANTCLF1* and *2*. The domain was named after the three founding members, Suppressor of variegation 3-9 (Su(var)3-9) (Tschierch *et al*, 1994), E(z) (Jones and Gelbert, 1993) and Trithorax (trx) (Chinwalla *et al*, 1995). All three of these *Drosophila* proteins are implicated as regulators of chromatin higher-order structure. Su(var)3-9 has a role in the silencing of heterochromatin, E(z) in the heritable silencing of homeotic genes and other targets, and trx which has an

opposite role. Trx is a member of the Trithorax group of genes (trx-G) that maintain heritable activation of homeotic and other targets during development. A number of additional SET domain proteins have been identified since this initial classification was made. All of these additional SET domain members also appear to be chromatin-associated and involved in epigenetic phenomena. These include: mammalian, plant and *C. elegans E(z)* homologues (Laible *et al*, 1997; Goodrich *et al*, 1997; Grossniklaus *et al*, 1998; Holdeman *et al*, 1998); the *ALL-1* gene, a human homologue of *Drosophila trithorax* that is involved in acute leukaemia (Rozovskaia *et al*, 2000; Rozenblatt-Rosen *et al*, 1998); and *clr4* a fission yeast gene with a role in silencing at mating type loci (Ivanova *et al*, 1998). In *Saccharomyces cerevisea* the SET domain protein SET1 is involved in telomeric silencing (Nislow *et al*, 1997).

The presence of the SET domain in many chromatin regulators implies a role in chromatin controlled gene regulation. Evidence for an ancient and evolutionarily conserved role in chromatin-mediated silencing was demonstrated by experiments showing that both human *EZH2* and murine *Ezh1* were able to restore the telomeric silencing in *Saccharomyces cerevisea* mutants deficient for the *SET1* SET domain gene (Laible *et al*, 1997). The same authors classified SET domain proteins into four subgroups based on sequence identity within this 130 amino acid domain. ANTCLF1 and 2 fall into the E(z) subgroup (Laible *et al*, 1997). A biochemical function for the SET domain in modifying chromatin has recently been discovered by Rea *et al* (2000). Using the SET domain genes human *SUV39H1* and murine *Suv39h1,* they demonstrated SET domain dependant methyltransferase activity. Specific

methylation occurred at lysine 9 of the N-terminus of histone H3 *in vitro*. In addition this specific histone methylation has recently been shown to create a binding site for the HP1 protein, a chromo-domain protein implicated in heterochromatic gene silencing (Lachner *et al*, 2001; Bannister *et al*, 2001).

The SET domain alone was not sufficient for H3-specific methyltransferase (HMT-ase) activity but required additionally an adjacent N-terminal cysteine rich region and a cluster of Cysteine residues c-terminal to the SET domain. Although E(z) and its homologues, contain the N-terminal cysteine rich region they lack the cysteines c-terminal to the SET domain. Consistent with this, the authors confirmed that human EZH2 did not have HMT-ase activity *in vitro i.e.* it did not methylate any of histones H1, H2A, H2B or H3 at any position. Therefore HMT-ase activity is only a characteristic of a subset of SET domain genes. As *E(z)* homologues neither *Arabidopsis CLF* or *Antirrhinum ANTCLF* genes (Figure 3.4b) contain the C-terminal tail necessary for HMT-ase activity. It is suggested in this paper that the subset of SET domains lacking HMT-ase activity may conversely intrinsically lack Mtase activity and that this may explain the seemingly contradictory roles of some SET domain genes.

E(z) related proteins are characterised by conserved cysteine rich domains with a unique spacing of cysteines .The functional importance of both these domains has been demonstrated in *Drosophila* E(z). Single Cys to Tyr substitutions in the first and second cysteine rich domains result in null *E(z)* alleles (Carrington and Jones,

1996). The second cysteine rich domain of ANTCLF1 and 2 has been particularly well conserved, this domain shares over 90% amino acid identity with *Arabidopsis* CLF.

The plant developmental gene *TSO1* has two cysteine-rich repeats (named TCR repeat 1 and 2) which are both similar to a portion of the 2nd cysteine rich region of E(z) and plant E(z) homologues. When one of these highly conserved cysteines is replaced with a tyrosine a severe mutant *tso1* phenotype results. *TSO1* encodes a nuclear localised protein which is believed to regulate floral meristem cell division and inflorescence meristem organisation (Lui *et al*, 1997; Hauser *et al*, 2000; Song *et al*, 2000). The TCR repeats of *TSO1* have been implicated as a DNA binding motif since the presence of these TCR repeats in soybean CPP1 is required for its DNA binding activity (Cvitanish *et al*, 2000).

The same authors identify a four amino-acid sequence, DGSL, conserved between *Arabidopsis* E(z) homologues and TSO1 which is found at the end of the 2nd cysteine rich region and the second TCR Repeat 2, respectively. These four amino acids are completely conserved in both TSO1 and the *Arabidopsis* E(z) homologues CLF and CLF-LIKE although MEDEA has the amino acid sequence DGTL instead of DGSL. It is therefore interesting that the sequence and position of the amino acid sequence DGSL has also been conserved in the *Antirrhinum* CLF homologues ANTCLF1 and 2. The conservation of this sequence is more significant because none of the other plant genes identified as having a TCR repeat 2 cysteine rich region also

have this sequence. It is possible that the conservation of this sequence indicates a common function of plant *E(z)* homologues and *TSO*. In addition the DGSL sequence is present within a 38 amino acid stretch, between the 2nd cysteine rich domain and the SET domain, that has been highly conserved between *Arabidopsis* CLF and *Antirrhinum* ANTCLF1 and 2 (87% identity, Figure 3.3b). This region is also well conserved between CLFLIKE and CLF but has not been well conserved between CLF and MEDEA, which does not contain the full DGSL motif.

Drosophila E(z) protein has been localised to embryonic and larval nuclei *in vivo* (Carrington and Jones, 1996). Similarly, the plant E(z) homologue MEA was shown to be nuclear localised in transfected plant cells (Spillane *et al*, 2000) and CLF protein has been identified as being nuclear localised in floral organs (L. Primavesi personal communication). *ANTCLF1* and *2* encode proteins which share the bipartite nuclear localisation domain present in both CLF and MEDEA, and therefore demonstrate the potential to be nuclear localised.

CLF contains a binding site for the retinoblastoma (Rb) protein and both maize and human Rb proteins have been show to interact with CLF in a yeast two-hybrid assays (Williams and Grafi, 2000). The Rb 'pocket' region which has been shown to be necessary for this interaction has been particularly well conserved between animal and plant Rb members (Xie *et al*, 1996). The putative Rb binding motif (LXCXD) which is found towards the N-terminus of CLF and CLFLIKE is surrounded by acidic residues. The binding site is not found in all plant E(z) homologues however,

MEDEA does have an acidic region in this position but this has been shown to be similar to the *trx* protein acidic region and there is no Rb binding site. The same putative Rb binding site as CLF is present within ANTCLF1 and 2, within a region which shares high sequence homology to CLF and CLFLIKE (Y. Chanvivatana personal communication, Figure 3.1, 3.2 and 3.3b) suggesting possible interaction with a yet unidentified *Antirrhinum* Rb protein.

The Rb protein is a key regulator of the cell cycle. The interaction of the Rb protein with the E2F transcription factor prevents the activation of E2F-target genes necessary for the progression of the cell into S-phase. Rb is believed to bring about the repression of these targets by modifying higher-order chromatin structure to a more repressive closed conformation. It may be that the plant Pc-G protein CLF contributes in this necessary chromatin modification by interaction with a promoter bound E2F-Rb complex. It is interesting that this binding domain has been well conserved in a subsection of *Arabidopsis* E(z) homologues and also distantly related *Antirrhinum* E(z) homologues but is not present in animal E(z) proteins. This suggests that the Rb interaction may provide a crucial functional difference between plant and animal E(z) homologues.

Although ANTCLF1 and 2 showed a high level of sequence similarity to CLF they were more similar to each other than either was to CLF, suggesting that the two *Antirrhinum* genes arose from a recent gene duplication. The expression of *ANTCLF1* and *2* also highlighted the high similarity of these two genes.

3.5.2 Expression of *ANTCLF1* and *2* is consistent with a role during both vegetative and floral development

ANTCLF 1 and *2* are expressed within the meristem and leaf primordia in common with the vegetative expression domains of *Arabidopsis CLF*. Both *ANTCLF* genes are expressed across the shoot meristem and in young leaf primordia after initation at the P_1 stage. Expression of *ANTCLF1* and *2* expression continues in leaves through later primordia stages to maturity. Weaker expression is also detected in the vasculature of the stem.

The expression of *ANTCLF* genes during floral development is also very similar to the expression pattern of *CLF*. Both genes are expressed in the inflorescence meristem, bracts and floral meristems. Expression is concentrated to the upper cell layers across the inflorescence meristem and is weak in the provasculature of the stem. *ANTCLF* gene expression was also seen in bract primordia, expression persisting up to the later stages of bract development but at a lower level. In floral meristems, which arise on the flanks of the inflorescence apex, *ANTCLF1* and *2* RNA is detected from stage 1 onwards. Expression can be detected in sepal primordia which become apparent at stage 2-3, and throughout all four developing floral organ primordia by stage 6. Expression then decreases in the maturing sepals and petal primordia, becoming strongest in the stamens and ovules in mature wild-type flower buds.

In summary both genes are expressed within the meristems and primordia of vegetative and floral tissue. The expression suggests a potential role for *ANTCLF1* and *2* during vegetative and floral development. The role of *Arabidopsis CLF* is the repression of the *c* function floral homeotic gene *AG*. It is possible that *Antirrhinum ANTCLF* genes have a parallel role in the repression of *Antirrhinum c* function genes although this cannot be established until *antclf* loss of function mutants have been identified. The early expression of *ANTCLF1* and *2* in the inflorescence meristem and floral meristems is consistant with a role in the regulation of floral homeotic genes. In common with *ANTCLF1* and *2, CLF* is expressed throughout all four floral whorls, indeed CLF protein has been identified in the nucleus of all four floral whorls (L. Primavesi personal communication). Contrastingly it is only required to repress *AG* in whorls 1 and 2 from stage 9 onwards. It is possible that interaction with regionally specific factors enables *CLF* to act in a temporally and spatially restricted manner within its zones of expression. This explanation could also apply to the *ANTCLF* genes, indicating the possibility of specific spatially and temporally restricted roles despite ubiquitous and persistent expression.

3.5.3 The function of Pc-G genes in *Antirrhinum*

The identical expression domains of *ANTCLF1* and *2* together with high sequence identity suggests that the genes may be functionally redundant. High levels of gene

duplication have been identified by analysis of the *Arabidopsis* genome, and single insertional mutants from these duplicated genes often have no discernable phenotype due to redundancy of function (reviewed in Martienssen and Irish, 1999). The possible redundancy of *ANTCLF1* and *2* means that a double mutant may be required to reveal a mutant phenotype and discover the true function of these genes. The alternative approach to discern gene function involves the analysis of transgenic plants (e.g overexpression analysis) but again the presence of functional redundancy and the presence of large gene families with overlapping functions can obscure the true function of genes within regulatory pathways. The floral homeotic MADS box genes of both *Antirrhinum* and *Arabidopsis* are characterised by a high level of redundancy. Analysis of the *FARINELLI (FAR)* gene, one of two *c* function floral homeotic genes of *Antirrhinum,* was obscured in ectopic expression analysis of transgenic plants, and only revealed after the isolation of a mutant by a reverse genetic screen and the production of a double mutant with *PLENA (PLE)* another *c* function floral homeotic gene (Davies *et al*, 1999). However in some cases redundancy does not obscure loss of function mutant phenotype, for example in the *ple* single mutant, despite redundancy with *FAR* (Davies *et al*, 1999).

These considerations meant that a reverse genetic approach was attempted to detect loss of function mutants of *antclf1* and *2*. PCR screening of transposon induced lines was used to look for insertions in *ANTCLF1* and *2*. Despite a thorough search, including the identification of a couple of candidate bands in first round screens, this method proved unsuccessful. Insertions towards the 5' end of *ANTCLF1* may not

have been identified because the lack of 5' sequence early in the project prevented the production of a 5' primer.

As an alternative approach candidate mutants which showed altered *c* function were analysed to determine if they represented *antclf1* and/or *antclf2* mutants . The *clf* mutant flower is characterised by partial homeotic transformation of the sepals and petals towards carpels and stamens respectively. Other morphological differences have been identified including reduced sepal curvature leading to premature opening of the flower bud and petals which are smaller and more narrow (Goodrich *et al*, 1997). These floral features are seen in the *Antirrhinum* mutants *heptandra (hep)* and *fistulata (fis).* Both have reduced and stamenoid petals, in addition the floral buds open prematurely (McSteen, 1997; McSteen *et al* 1998; Motte *et al*, 1998). The partial homeotic transformation of the petals of *fis* and *hep* result from the ectopic expression of *PLE* which has been detected at stage 5 and stage 6 respectively (McSteen *et al*, 1997; McSteen *et al*, 1998). Ectopic expression of *AG,* the homologous *Arabidopsis c* function gene, is also detected in the petals of *clf-2* mutants if at a later stage of development, *Arabidopsis* stage 9. Therefore *fis* and *hep* represented strong candidates for *antclf1* and/or *antclf2* mutants.

Southern analysis was used to reveal whether either of these mutants had resulted from transposon insertion into *ANTCLF1* or *2*. It was concluded that the *hep* mutant did not result from a transposon insertion into *ANTCLF1* or *2*. More subtle gene alterations resulting from an initial transposon insertion that excised imprecisely, or

small mutations unrelated to transposon insertion, could not be ruled out since linkage analysis was not tested. The Southern blot analysis also indicated that transposon insertion into *ANTCLF1* or 2 was an unlikely causal factor for the *fis* mutant, however the small sample used and inconsistencies in the Southern analysis preclude a firm conclusion. Again linkage analysis was not successful. Suitable RFLPs between the relevant *Antirrhinum* parental lines have been identified by Nina Ehrenberg for *ANTCLF2* analysis and during the course of this study RFLPs have also been identified which could be used in future for *ANTCLF1* analysis.

Other factors make it unlikely that *fis* and *hep* represent *antclf* mutants, these include their status as single recessive mutants. Genetic analysis of segregating *hep* families conducted during this study reinforce previous speculation that *hep* is a single recessive mutant (McSteen *et al*, 1997), *fis* is also a recessive single mutant (McSteen, 1997). If *ANTCLF1* and *2* are functionally redundant a single mutant would not be expected to have a distinct phenotype, both *fis* and *hep* have a noticeable phenotype. Double mutants with similar phenotypic characteristics are more likely to represent an *antclf1/antclf2* double mutant.

Another candidate for an *antclf* mutant was *polypetala (poly),* a semi-dominant mutant. Heterozygous *poly* flowers have an extra whorl of petals whereas the flowers of homozygous *poly* are characterised by an indeterminate number of petals. These phenotypic changes result from a reduction in *ple* expression in the central dome of the floral meristem (McSteen *et al*, 1998). Since *poly* is semi-dominant it is possible

that it is a gain of function mutation, this would mean that the wild-type role of *poly* was the down regulation of *PLE* in the outer floral whorls and in the gain of function allele this activity extends to whorls 3 and 4. Southern blot analysis showed, however, that *poly* does not result from a transposon insertion into *antclf1* or 2. In addition linkage analysis showed that *poly* does not result from smaller mutational changes in *ANTCLF2* as the two locus are unlinked genetically. Another factor which suggests that *poly* may not result from an *ANTCLF* mutation concerns the early differences in *PLE* expression in the *poly* homozygous flower. Reduction in *PLE* is detected in the centre of the floral meristem by stage 4 suggesting that *poly* effects the initial pattern of *PLE* expression (McSteen *et al*, 1998). The Pc-G genes *ANTCLF1* and *2* would be unlikely to have such a role since *CLF* and animal Pc-G genes act only to maintain the silencing of targets and do not initially specify the repression of targets.

The last candidate mutant to be tested, *carnosa (car)*, was chosen because of its vegetative phenotype. *car* has small rolled leaves making it similar to *clf* which has small upward curling leaves. It is not certain that the *antclf* mutants would have curled leaves even if they did show misregulation of *c* function, because in the *ovulata* mutant the *c* function floral homeotic gene *PLE* is ectopically expressed in leaves but this does not result in leaf curling but rather in occasional crinkling and deformation of the bracts (Bradley *et al*, 1993). Southern blot analysis suggested that the *carnosa* mutant was not caused by a transposon insertion into an *ANTCLF* gene, however lack of the predicted RFLP suggested that the cross had not used JI98 as

expected. Further Southern blot analysis should be undertaken to confirm that *car* does not represent an *antclf1* or *antclf2* mutant.

As a third approach to determine the function of *ANTCLF1* and *2* viral-induced gene silencing was attempted, to reduce or eliminate expression of the two genes. This represented the first time that viral-induced gene silencing had been attempted in *Antirrhinum*. The tobacco rattle virus (TRV) was chosen because it has been shown to have a wide host range (MacFarlene and Popovich, 2000) and because it is able to infect meristematic regions where the *ANTCLF* genes are expressed.

Viral constructs were produced which contained non-conserved sequence of *ANTCLF1* and *ANTCLF2* in the hope of producing plants where each gene had been silenced. In addition highly conserved *ANTCLF1* sequence, sharing 86% amino acid identity with *ANTCLF2,* was used in a seperate viral construct with the aim of silencing both genes. All three viral constructs were able to infect within the innoculated leaf but only the viral construct pK_2OG1C, containing conserved *ANTCLF1* sequence, showed signs of localised systemic spread. No discernable phenotype was observed to indicate viral-induced silencing within any infected plant. In addition gene specific RT-PCR failed to detect reduction in *ANTCLF1* or *2* expression within these plants.

These results were disappointing especially since TRV has recently been shown to be efficient in a VIGS system with a number of advantages over previously used virus's

(Ratcliffe *et al*, 2001). TRV-induced silencing in *Nictotiana* species was shown to provide more persistant silencing of some targets, and in addition affected a higher proportion of tissue. Initial experiments with *Arabidopsis* also indicated the potential for development of future TRV-VIGS silencing programs. More importantly the TRV VIGS system was successful in silencing *Nicotiana FLO/LFY* (NFL) the homologue to the *Antirrhinum FLORICAULA* gene and the *Arabidopsis LEAFY* gene. Previous attempts to silence this gene using a potato virus X (PVX) failed because this virus cannot infect growing points (Angell and Baulcombe, 1999). TRV was shown to effectively infect growing points and silence NFL, producing plants with secondary and tertiary branches and abnormal flowers resembling those seen in *flo* and *lfy* (Ratcliffe *et al*, 2001). PVX has been shown to encode a movement protein, this is thought to prevent the systemic spread of a RNA-mediated silencing signal (Voinnet *et al*, 2000). Only after this movement protein had been removed could long-distance spread of silencing occur in VIGS experiments. This may have been suggested as a possible reason for the lack of silencing observed in this report, however Ratcliffe *et al* (2001) state that the TRV system is not affected by movement protein restrictions.

The isolation of *Antirrhinum antclf1* and *2* mutants in the future will enable the Pc-G function of two distantly related plant species to be compared, providing valuable insight to Pc-G function and chromatin regulation in the plant kingdom. The results of this study have demonstrated the similarity in sequence and expression domains

between the *ANTCLF* genes and *Arabidopsis CLF*. This conservation suggests that *CLF* homologues have an important function throughout the plant kingdom.

CLF is not the only *E(z)* homologue in *Arabidopsis,* two other homologues have been identified namely *CLFLIKE* and *MEDEA (MEA)* (Grossniklaus *et al*, 1998; J Goodrich personal communication). *MEA* has a different function from *CLF,* regulating cell proliferation during seed development. It is therefore possible that additional *E(z)* homologues exist in *Antirrhinum* and that these have different roles from *ANTCLF1* and *2*. Further reducing the stringency of *Antirrhinum* genomic and cDNA library screens using a *CLF* probe may enable these to be identified.

MEA interacts with another Pc-G protein FIE, the *Arabidopsis* homologue of *Drosophila Extra sex combs (Esc)* (Spillane *et al*, 2000). Yeast two-hybrid analysis has shown that the same interaction occurs between CLF and FIE (J Goodrich, C stock pers comm). In both *C. elegans* and *Arabidopsis, E(z)* and *Esc* homologues represent the only identified members of the Pc-G family (Xu *et al*, 2001; Spillane *et al*, 2000). Therefore, it is likely that a similar complex exists in *Antirrhinum*. An N-terminal domain of E(z) and its homologues has been shown to interact with Esc homologues in both animals and plants (Spillane *et al*, 1999; Jones *et al* 1998; van Lohuizen *et al*, 1998; Sewalt *et al*, 1998). Although this region has not been conserved between animals and plants both MEA and FIE can interact with *Drosophila* Esc in a two-hybid assay (Spillane *et al*, 2000). The region of MEA sufficient for interaction with FIE (amino acids 4-109) has also not been well

conserved amongst the *Arabidopsis E(z)* homologues *CLF, MEA* and *CLFLIKE* therefore it is not possible to identify a FIE interaction domain in the ANTCLF amino acid sequence.

Other features of *Antirrinum* make it an ideal plant species for the study of Pc-G function. Firstly the floral homeotic genes of *Antirrhinum* have been well characterized. Two *c* function floral homeotic genes have been identified in *Antirrhinum FAR* and *PLE* (Davies *et al*, 1999; Bradley *et al*, 1993). The *ANTCLF* genes may regulate either or both of these, *AG* is most closely related to *FAR* although *PLE* has an organ specification role more similar to *AG*. Secondly the isolation of *antclf* mutants might help to determine whether Pc-G genes have a role in the regulation of dorsoventral patterning of the *Antirrhinum* flower. Pc-G regulation of one of the main floral dorsoventral regulators *CYCLOIDEA (CYC)* has been suggested by work on the semi-dominant *backpetals* allele of *CYC* (Luo *et al*, 1999). In *backpetals* silencing of *CYC* breaks down but only late in development. It has been suggested that the transposon insertion responsible for *backpetals* disrupts a cis-acting regulatory region of *CYC* that is normally regulated by a Pc-G protein explaining why early expression is unaffected.

In future *ANTCLF1* and *2* function might be studied by transforming *Antirrhinum* as this method is now possible. Locating the *ANTCLF* genes on an *Antirrhinum* RFLP map may also indicate further candidate mutants such as *stylosa.*

4. Use of a steroid inducible CLF+ line to determine the developmental time of *CLF* silencing

4.1 Introduction

The *Arabidopsis CLF* gene has an important role in the repression of *AG* so that in *clf-2* mutants *AG* is misexpressed in the leaves, inflorescence stem and flower (Goodrich *et al*, 1997). *CLF* RNA is expressed ubiquitously and persistently during development. In the flower *CLF* RNA is present in all floral whorls throughout development, however it only acts at late floral stages to repress *AG* in a subset of the whorls (whorls 1 and 2). This result demonstrates that the time and sites of *CLF* action cannot be determined by studying expression alone. This chapter describes the use of a transgenic line conferring steroid inducible CLF+ activity, to determine the developmental time of *CURLY LEAF (CLF)* action.

CLF is the homologue of the *Drosophila Enhancer of Zeste* (*E(z)*) which encodes a Pc-G repressor protein believed to act by modifying higher order chromatin structure. Pc-G mediated silencing in animals is heritable as cells undergo many

cycles of division after the period in early embryogenesis when patterns are generated by transiently expressed regulators. The molecular basis of heritable silencing is not well understood but conceptually is believed to involve a heritable 'tag' which marks the repressed target gene through the disruptions of DNA replication, and during chromatin condensation in late prophase/metaphase when a number of Pc-G proteins have been shown to dissociate from chromatin (Buchenau *et al*, 1998). The heritable "tag", which is believed to involve some form of DNA or chromatin modification, must also be able to propagate itself. An example of a possible propagating heritable "tag" is DNA methylation, the semi-conservation mechanism of DNA replication produces hemi-methylated DNA which is then detected by methyl transferases and methylated on the daughter strand. This type of heritable "tag" might be independent of Pc-G products after it had formed, thus the Pc-G protein involved may only be required transiently to "set up" the heritable tag. An example of a Pc-G gene which is only required transiently is *Extra sex combs (Esc)* (Simon *et al*, 1995). Alternatively the heritable "tag" may be dependent on the Pc-G activity in which case the Pc-G protein would be required continuously to maintain heritable silencing.

The majority of Pc-G gene products are required continuously to maintain homeotic gene repression, including *E(z)* (Jones and Gelbart, 1990; Phillips and Shearn, 1990; Shearn *et al*, 1978). CLF may be required continuously throughout development to repress its targets, as is the case for its *Drosophila* homologue *E(z)*. Alternatively it may only be required transiently, producing a heritable epigenetic tag that is

maintained by other factors. Studying the time during development when CLF+ is necessary or sufficient for silencing *AG* may help clarify possible mechanisms of action.

A transgenic line conferring steroid inducible CLF+ activity was used to investigate the temporal requirement for *CLF*. Steroid inducible systems have been used successfully to study a number of transcriptional activators in *Arabidopsis* (Lloyd *et al*, 1994; Simon *et al*, 1996; Sablowski and Meyerowitz, 1998; Wagner *et al*, 1999; Samach *et al*, 2000). *CLF* is the first plant repressor that has been studied using a steroid inducible system. The system involves fusing the protein of interest to the Ligand Binding Domain (LBD) of the rat glucocorticoid receptor (GR). In the absence of the synthetic ligand analogue dexamethasone (DEX) the protein fused to the GR-LBD is bound by hsp90 proteins and inactivated. When DEX is applied the hsp90 proteins are released activating the protein of interest. If the protein of interest has a nuclear localisation signal it will now translocate to the nucleus.

Similar to temperature sensitive alleles or other conditional mutants, the steroid inducible system allows the activity of a gene to be switched on or off at specific developmental time points. Temperature sensitive *E(z)* alleles were extremely important for characterising the temporal requirement for *E(z)* and for identifying its function at different developmental stages, especially those beyond the early embryo lethal phase (Shearn *et al*,1977, Jones and Gelbert, 1990). However, the effect of shifting flies from a restrictive temperature to a non-restrictive temperature could not

be tested because absence of zygotic $E(z)^+$ product results in early pupal lethality (Shearn *et al*, 1977).

This chapter describes the results of shifting experiments designed to determine whether *CLF* is required at late stages of leaf development to maintain repression of *AG*. An *AG* reporter line was introduced into the steroid inducible CLF+ line to monitor *AG* expression in detail during the shifting experiments. To investigate whether *CLF* can repress *AG* in leaves where *AG* has previously been transcriptionally activated, seedlings were shifted from a media lacking DEX onto a DEX containing media.

4.1 The steroid inducible CLF+ construct p(CLF)-CLF::GR

The steroid inducible CLF+ construct was made by J. Goodrich. A 7 kb *Not* I/*Kpn* I fragment of genomic DNA that spans the *CLF* locus was previously found to complement null *clf* mutations (J. Goodrich personal communication). A 1.1 kb *Xba* I/*Eco* RI fragment from the plasmid pBI-ΔGR (a gift from Alan Lloyd) encoding the rat Glucocorticoid receptor Ligand Binding Domain (GR-LBD), was cloned into *Xba* I/*Eco* RI sites introduced into the final exon of the *CLF* gene. The resulting construct expresses an in-frame fusion of the GR-LBD to the C-terminus of the CLF protein, under control of the *CLF* promoter (Figure 4.1).

To test whether the construct gave steroid dependant rescue of CLF it was introduced into a *clf-* background. The construct was inserted into the pGreen binary Ti vector pGR0229 for *Agrobacterium tumefaciens* mediated plant transformation (Hellens *et al*, 2000). The pGR0229 vector carries a BAR gene, which confers resistance to the herbicide BASTA, as a selectable marker for plant transformation. Both the *CLF::GR* construct and the BASTA herbicide resistance selectable marker were placed between the right and left T-DNA borders of the plasmid for random insertion into the genome during transformation.

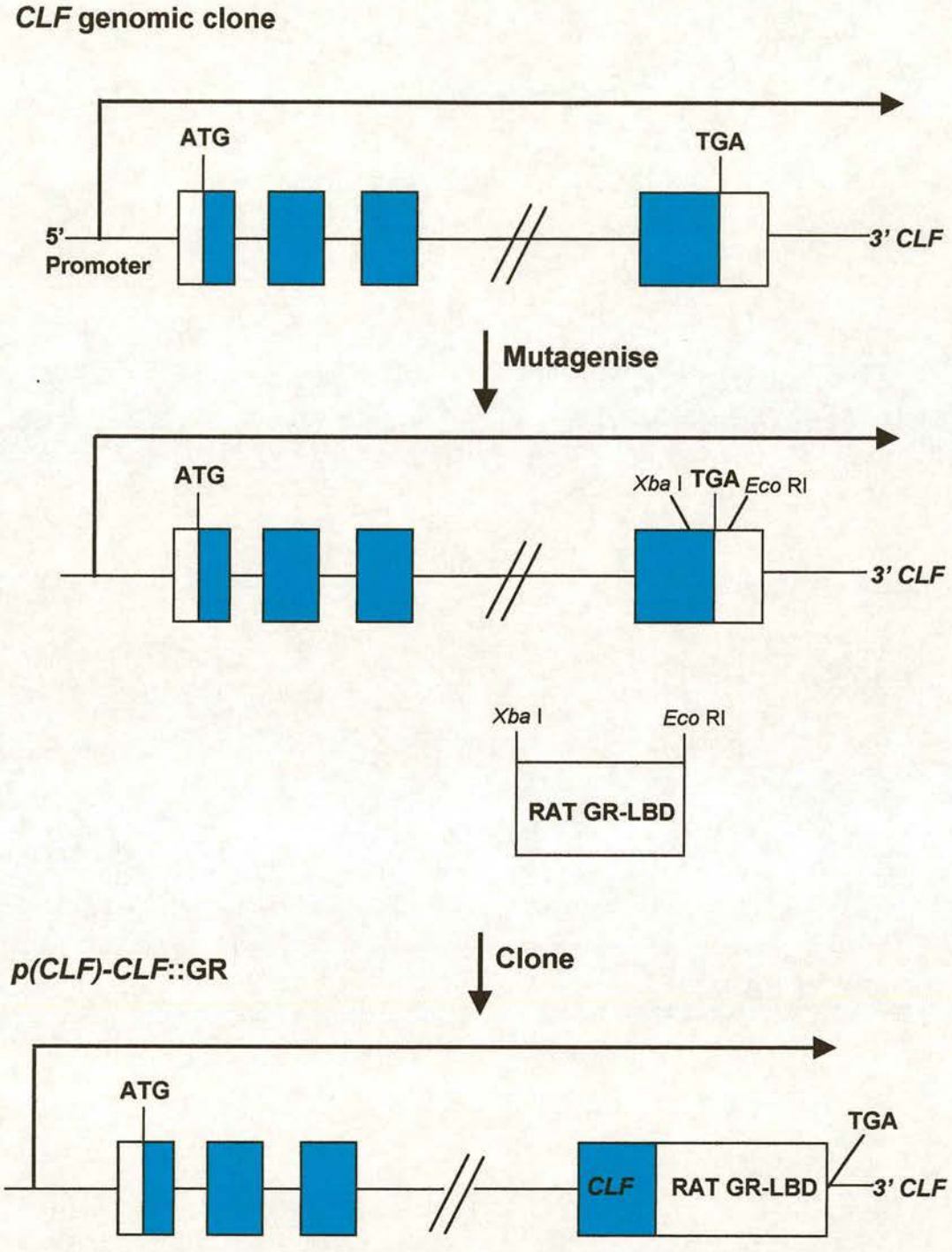


Figure 4.1 Schematic diagrams to show the production of a steroid inducible CLF+ construct *p(CLF)-CLF*::GR. 7 kb of genomic *CLF* (including the 5' promoter) was used to produce the construct. Restriction sites were introduced into the final exon of the *CLF* gene to allow a 1.1 kb fragment encoding the GR-LBD to be cloned as an in-frame fusion. The CLF promoter directs expression of a CLF::GR fusion protein. *p(CLF)-CLF*::GR was made by J. Goodrich.

Wild-type *clf-2/+* heterozygotes were transformed using the floral dip method (Clough and Bent, 1998). In the T1 progeny 10 independent transformants were identified using herbicide (BASTA) selection. From these, two *clf-2/clf-2* lines were obtained which were homozygous for single locus insertion of the p(CLF)-CLF::GR construct. These lines, named I36 and I38 were used in subsequent experiments.

4.2 Steroid dependent rescue of *clf-2* in transgenic *CLF::GR* lines

It was important to demonstrate that the CLF::GR fusion protein had steroid dependent CLF+ activity. Before testing the transgenic lines I36 and I38, wild-type and *clf-2* mutant plants were grown in the presence of DEX to test whether the steroid treatment alone had any effect. DEX was used at a concentration of 10 μM both in GM tissue culture media and in solutions used for watering and spraying soil grown plants. This DEX concentration had previously been used effectively to induce soil grown *CO::GR* plants by Simon *et al* (1996). The DEX treatment had no effect on the morphology of either the wild-type (Figure 4.2a) or *clf-2* mutants (Figure 4.2b). Previous studies involving a steroid inducible approach, have also shown that hormone treatment has no effect on *Arabidopsis* growth (Simon *et al*, 1996; Wagner *et al*, 1999; Sablowski and Meyerowitz, 1998).

Steroid dependent rescue of the *clf-2* phenotype occurred when the I38 line was germinated and grown in the presence of DEX. In the absence of DEX these plants have a rosette of small curled leaves. In contrast, when grown in the presence of DEX the leaf rosette appears as wild-type (Figure 4.2c). Other aspects of the *clf-2* phenotype such as early flowering and short stem length showed morphological rescue when the I38 line was grown on DEX.

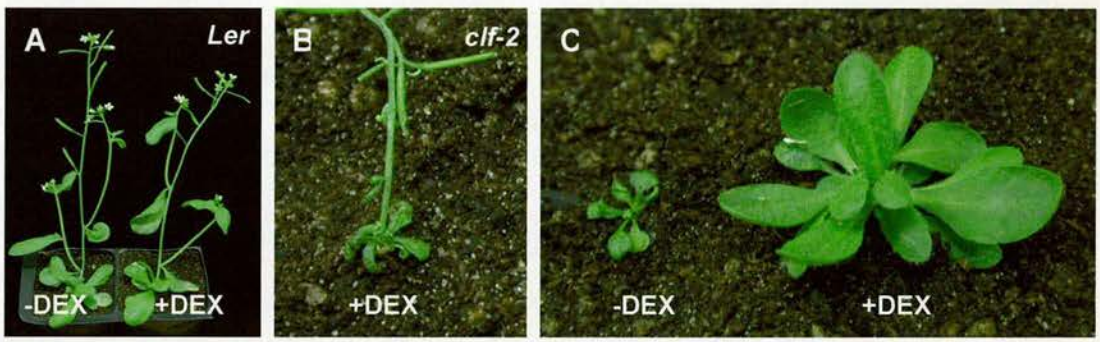


Figure 4.2 Steroid dependant rescue of *clf-2* in transgenic plants with steroid inducible CLF+ activity. DEX treatment had no effect on the morphology of wild type (*Ler*, A) or *clf-2* mutant plants (B). In contrast phenotypic rescue of the *clf-2* phenotype was observed when plants of the I38 line were grown in the presence of DEX (C).

It was important to establish whether the phenotypic rescue of plants treated with DEX reflected the repression of *AG* by functional CLF::GR fusion protein. Analysis of products produced from polymerase chain reaction after reverse transcription (RT PCR) was used to compare the *AG* expression levels present in I36 and I38 lines grown in the presence and absence of DEX .Total RNA was extracted from 14-day-old seedlings. Each seedling was dissected before RNA preparation so that the RNA present in the leaves and cotyledons could be extracted separately. The RT PCR revealed clear differences in *AG* expression between samples grown in the presence of DEX and those grown without DEX (Figure 4.3). In the absence of DEX strong *AG* expression was detected in leaf and cotyledon fractions from both lines. In contrast little or no *AG* was present in the leaves or cotyledons fractions of DEX treated plants. These results confirmed that the morphological rescue observed when *CLF::GR* plants were treated with DEX resulted from an activated CLF::GR fusion protein capable of repressing *AG*.

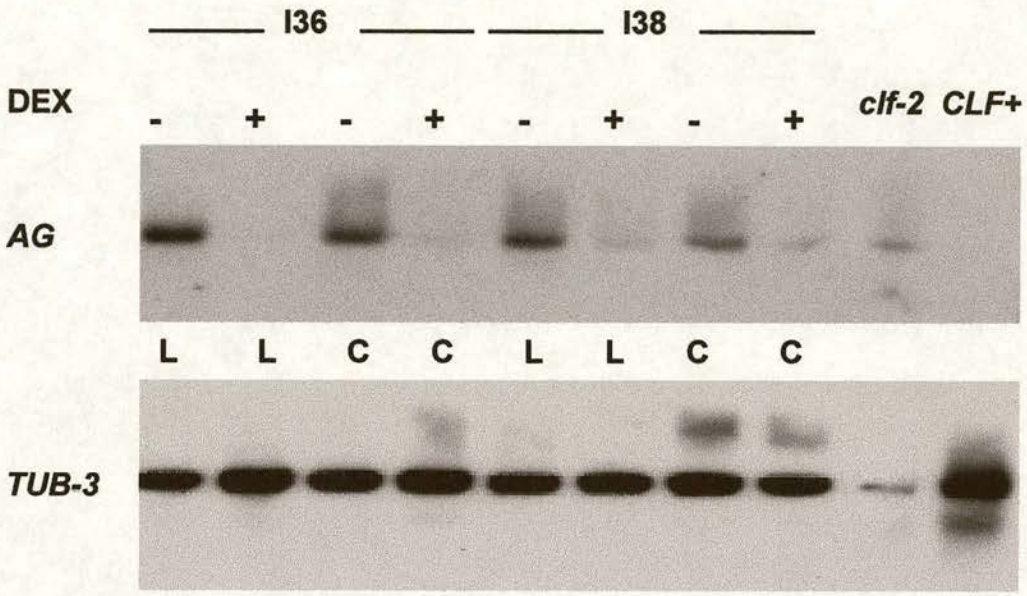


Figure 4.3 RT PCR analysis of *AG* expression in the steroid inducible CLF+ lines I36 and I38 Seedlings were grown in the presence or absence of DEX. At 14 days each seedlings was dissected above the cotyledons to separate the cotyledons from the leaves. RNA was extracted from pooled cotyledons **C** and leaves **L**. After non-maximal RT-PCR, the PCR products were subjected to electrophoresis on agarose gels and then transferred to Nylon membranes to be probed with an *AG* DIG probe. *AG* is expressed in both the **C** and **L** fractions when I36 and I38 are grown in the absence of DEX, but expression is weak or absent in these fractions when grown on DEX. β*TUBULIN-3 (TUB-3)* was used as a control for equal loading and amplification. *CLF+* (*Ler)* and *clf-2* were also analysed as controls for differential *AG* expression.

4.3 Introduction of an *AG* reporter into the steroid inducible CLF+ line

An *AG* reporter was introduced into the I38 background to facilitate analysis of *AG* expression during shifting experiments. The pAG-I::*GUS* reporter construct was used (Sieburth and Meyerowitz, 1997). The construct includes 3.8 kb of intragenic sequence in addition to 6 kb of upstream sequences. The intragenic sequences contain regulatory sites which are essential to replicate the normal pattern of *AG* expression. Sieburth and Meyerowitz (1997) introduced pAG-I::*GUS* and another construct lacking intragenic region (pAG::GUS) into *clf-2/clf-2* and CLF+ backgrounds and showed that *CLF* is able to regulate the pAG-I::*GUS* construct correctly.

Initially the pAG-I::GUS construct was introduced into CLF+ and a *clf-2* mutant background to observe in detail the differences in patterns of GUS activity. No staining is present in the leaves and cotyledons of wild-type CLF+ plants which contain the pAG-I::*GUS* construct, due to repression by *CLF* (Figure 4.4a). In contrast GUS activity is clearly visible in the leaves and cotyledons of *clf-2* mutants containing pAG-I::*GUS* (Figure 4.4b).

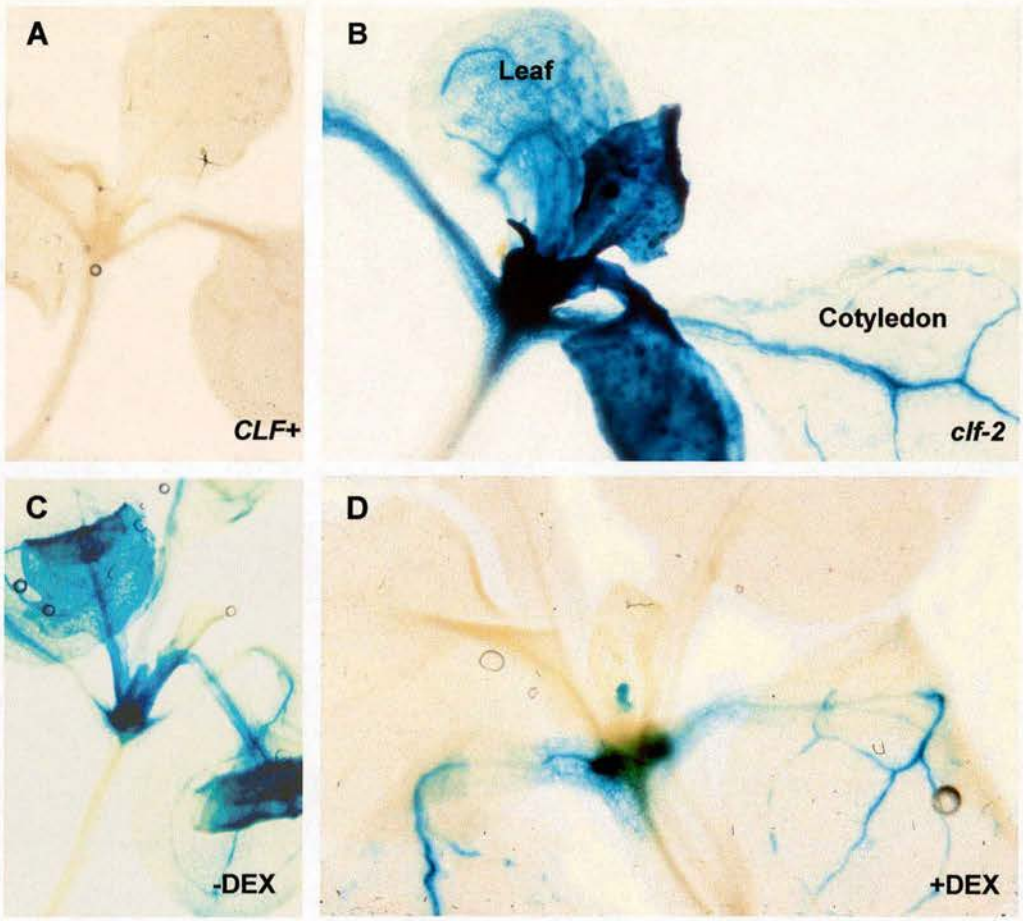


Figure 4.4 Monitoring *AG* expression in the steroid inducible CLF+ line I38 using the pAG-I::*GUS* reporter construct. Gus expression in wild type (A) and *clf-2* mutant (B) lines containing pAG-I::*GUS*. GUS expression is absent from the leaves and cotyledons of wild type plants due to repression of *AG* by *CLF* (A), *AG* misexpression is detected as strong GUS activity in both the leaves and cotyledons of *clf-2* mutant plants (B). Plants of the steroid inducible CLF+ line containing pAG-I::GUS (the K57 line) were grown for 14 days in the presence (D) or absence (C) of DEX. Whole seedlings were treated with x-gluc. *AG* misexpression is detected as strong GUS expression in the leaves and cotyledons of an untreated plant (C). In contrast no GUS expression is apparent in the leaves of the DEX grown plant (D). Weak GUS expression is present in the cotyledons.

The pAG-I::*GUS* reporter line was introduced into the inducible CLF line I38 (Figure 3.5). To do this, the pAG-I::GUS *clf-2* line was crossed to the I38 line by J. Goodrich. In the F2 of the cross *clf-2/clf-2* plants that carried the inducible transgene, p(CLF)-CLF::GR, were selected using BASTA herbicide resistance. Resistant plants were then tested for presence of the pAG-I::*GUS* reporter by GUS staining of the flowers. Screening of the F3 progeny of lines identified F2 plants homozygous for both pAG-I::*GUS* and p(CLF)-CLF::GR. One *clf-2/clf-2* line was obtained which was homozygous for both transgenes. This line was named K57.

K57 plants were germinated and grown in the presence and absence of DEX on agar plates. As expected plants grown without DEX display strong GUS activity in the leaves and a weak veinous expression in the cotyledons similar to the pattern of misexpression seen in *clf2-/clf2-* plants (Figure 4.4c). *AG* is not present in the leaves of induced plants suggesting that *CLF* is induced and functioning normally (Figure 4.4d). This confirmed that steroid induction affected *AG* expression. Unexpectedly however weak veinous expression is present in the cotyledons of induced K57 plants, unlike in *CLF+* plants (Figure 4.4a). Because cotyledons form during seed development, unlike leaves which develop after germination, this may have reflected a lack of steroid induction during seed development This may have been because the parent plants grown to provide seed for the experiment had not been treated with DEX after the plants had flowered and the seed was developing. To test this hypothesis K57 plants were grown and treated with DEX throughout seed

maturation. K57 seeds were grown on DEX treated agar plates and later transferred to magenta's also containing DEX treated agar. The plants were supplied with DEX via the agar until the siliques had matured. These seeds were germinated and grown on DEX treated agar. K57 plants were also grown in the absence of DEX to act as controls, seeds were collected from these plants and grown on plates lacking DEX.

Figure 4.6 shows that *AG*-GUS misexpression is still observed in cotyledons which derived from seeds treated with DEX throughout their development (Figure 4.6b). This may be because the steroid cannot enter the seed through the seed coat, alternatively the p(CLF)-CLF::GR construct may lack the correct regulatory sequences required to implement *AG* repression in the cotyledons.

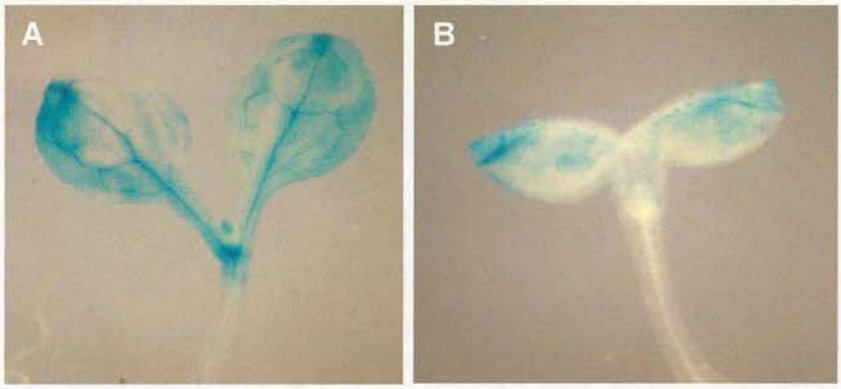


Figure 4.6 The effect of growing K57 plants on DEX throughout development including seed development. K57 plants were grown in the presence of DEX on plates and later in magenta containers in the presence of DEX until seed was mature. These seeds were germinated and grown on plates containing DEX. (A) Control plants were grown in the absence of DEX, seeds from these plants were collected and grown without DEX, *AG*-GUS expression is present in the cotyledons and leaf primordia. (B) *AG*-GUS expression is also present in the cotyledons of plants grown from seed which developed in the presence of DEX.

4.4 Shifting experiments

4.4.1 Effects on *AG* expression of withdrawing DEX in the K57 line

The K57 steroid inducible CLF+ line carrying pAG-I::*GUS* was used to facilitate monitoring *AG* expression during shifting experiments. *CLF* was inactivated at a particular developmental time by shifting K57 seedlings from DEX containing GM media to normal GM media lacking DEX. A previous experiment (J. Goodrich, unpublished data) suggested that *CLF* is needed persistently during leaf development. I38 plants were germinated and grown in the presence of DEX for 17 days and then transferred onto media lacking DEX. By 13 days after the shift some leaves were curled suggesting that they had began to misexpress *AG* as a result of the shift. In contrast the leaves of control seedlings grown on DEX throughout had a wild-type phenotype. Although this initial experiment suggested that *AG* became activated in leaves of shifted plants it could not reveal when or where this was occurring . For example, leaves that were well advanced in development at the time of the shift did not develop curling. However, they may have expressed *AG,* but this was without morphological consequences at late developmental stages. The K57 line containing the *AG* reporter was used to investigate the precise temporal and spatial

pattern of *AG*-GUS expression after shifting plants from inductive to non-inductive conditions.

An initial experiment was performed using a number of shifting time points. This would determine the time taken for misexpression to appear in plants shifted at different developmental times. Previous shifting experiment using steroid inducible systems have all involved transcriptional activators. Responses involving the activation of downstream targets have been rapid (within 2 hrs) when plants are treated with DEX (Samach *et al*, 2000; Wagner *et al*, 1999; Simon *et al*, 1996). The time taken for a response involving loss of *AG* repression after removal from DEX was difficult to estimate, because it is unclear how long DEX can persist in the plant after the shift.

Seedlings were grown on DEX for 4, 6, 9, 12, 15 or 18 days and then transferred onto plates containing normal GM media. Control plants were grown on DEX throughout the experiment. *AG* expression was monitored by staining a small number of seedlings for GUS activity on the day of the shift (S+0) and every second day (S+2, S+4 etc) until 16 days after the shift. A final staining reaction was carried out 21 days after the shift.

A number of days after shifting, GUS activity could be detected in the leaves demonstrating that *AG* had become misexpressed in shifted but not control plants. The time taken for the GUS expression to appear in the leaves correlated with the age

of the plant at the time of the shift (Table 4.1 below). GUS activity was detectable in the first pair of leaves from the day 4 sample 8 days after shifting. These leaves were not visible to the naked eye at the time of the shift and would have been young leaf primordia (Bowman, 1994), they displayed veinous GUS activity indicating weak misexpression of *AG*. Subsequent leaves (leaves 3, 4, 5 etc) showed stronger GUS expression indicating an increase in *AG* misexpression. In contrast negligible GUS expression was apparent in the leaves of control plants grown on DEX throughout. A similar pattern was observed for plants shifted at all of the time points. These results indicate that *CLF* is required persistently during leaf development to repress *AG*.

Age of K57 plants at the time of the shift (Days)	*AG* misexpression detected in leaves as GUS expression (Days after shift)
4	8
6	10
9	14
12	16

Table 4.1 The time taken for *AG*-GUS expression to appear in the leaves of K57 plants shifted off DEX at different developmental stages.

In some cases GUS activity was detected in leaves that would have been mature at the time of the shift. Seedlings shifted at 12 days had 3 to 4 visible leaves. GUS expression was absent from these leaves at the time of the shift. By day 16 GUS expression had become visible in all of the leaves from shifted samples. *CLF* would have been functional in the first 3 to 4 leaves until late in their development. Misexpression of *AG* in these leaves after the shift indicates that *CLF* is required throughout leaf development to repress *AG*.

The shifting experiment was repeated to confirm that *CLF* is required late/throughout leaf development to repress *AG*. Shifting was carried out at 9 and 12 days. These time points were used to enable *AG* expression to be monitored in leaves already mature at the time of the shift. At 9 days post-germination the first pair of *Arabidopsis* leaves will be entering the later expansion phase of their development. At the tip of the leaf, cells are differentiated and expanding whereas cells towards the base of the leaf are still in the earlier mitotic phase (Figure 4.11a, Bowman, 1994). The third and fourth leaf are also visible at 9 days and have enlarged by 12 days (Figure 4.11a and 4.1a).GUS expression was analysed on the day of the shift, the zero time point (S+0). The next GUS analysis was timed to take place after *AG* GUS leaf misexpression had become visible in the previous experiment, three later time points were also analysed. Table 4.2 shows when GUS analysis was carried out. All of the samples were germinated and analysed on the same days.

Age of plant	Time point identification	Time points for analysis of the 9 day shift (S = shift)	Time points for analysis of 12 day shift (S = shift)
9		S+0	
12			S+0
26	1	S+17	S+14
29	2	S+20	S+17
33	3	S+24	S+21
40	4	S+31	S+28

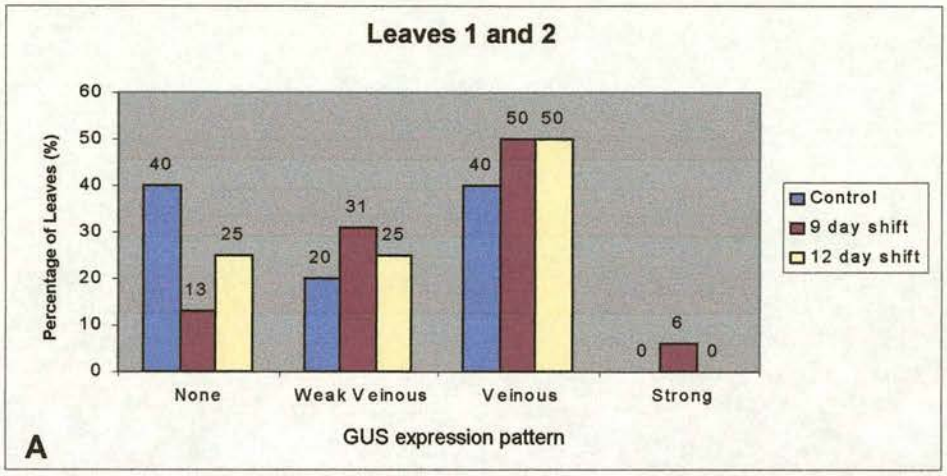
Table 4.2 GUS expression was analysed at the time of the shift and at four time points after the shift.

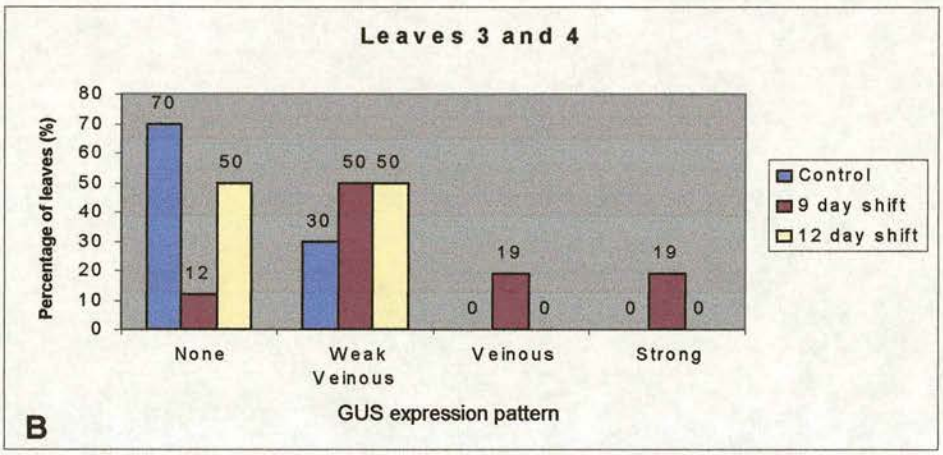
Over fifty seedlings were grown continuously on plates containing DEX to act as controls. Between 5 and 10 control seedlings were analysed together with the same number of shift samples at the first four time points. A larger sample was analysed at the last time point. At each time point the level of GUS activity in the first six leaves from shifted and control plants was analysed. The leaves could not be individually identified and numbered in some cases therefore GUS activity in each two leaves was determined. GUS activity in the first leaf pair was analysed as an average. The GUS activity in leaves 3 and 4 and lastly 5 and 6 was also considered as an average.

Three different levels of GUS expression were distinguished, a weak stain in the main veins, a strong veinous stain or strong staining throughout the leaf.

Histochemical analysis of GUS activity in DEX grown control plants revealed *AG* repression was only partial in the first pair of leaves. Some misexpression had also been noticed in this pair of leaves in the initial experiment. Weak GUS expression was present in 60% of these leaves at the first time point (Figure 4.7a) falling to 22% at time point three (Figure 4.9a) then rising again to 46% at the last time point (Figure 4.10a). The *AG*-GUS misexpression in these controls was always restricted to the veins and usually weak. In contrast repression was convincing in leaves 3, 4, 5 and 6, especially at time points 2 and 3 (Figure 4.8b and c) and (Figure 4.9b and c).

AG repression was also incomplete in the cotyledons which are embryonically derived, as discussed in 4.1.3. There is some evidence to suggest that the first pair of *Arabidopsis* leaves are also initiated embryonically (McConnell *et al*, 2001). Small bumps indicating the presence of the first two leaf primordia have been observed in SEM preparations of dissected late embryos (K.Barton, personal communication). *AINTEGUMENTA* which is expressed in young leaf primordia has been used to positively identify these primordia during late embryonic development *i.e.* two patches of *ANT* expression, corresponding to leaves 1 and 2 are observed between the cotyledons late in seed development (K.Barton, personal communication). The detection of GUS activity in the cotyledons and first pair of leaves may indicate that pAG-I::*GUS* is persisting in tissues formed during embryo development. *AG*





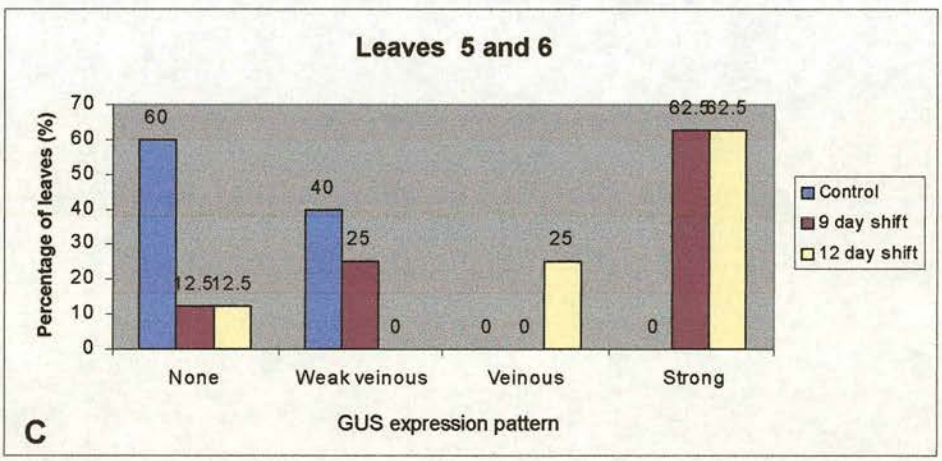


Figure 4.7 GUS expression levels in the leaves of K57 plants shifted off DEX compared to control plants at S+17 (9-day shift) and S+14 (12-day shift). GUS activity was analysed in the first six leaves of plants shifted at 9 days (n = 8) and 12 days (n = 4). Control plants (n = 5) were grown in the presence of DEX from germination and analysed on the same day. All plants were 26 days old at the time of the analysis.

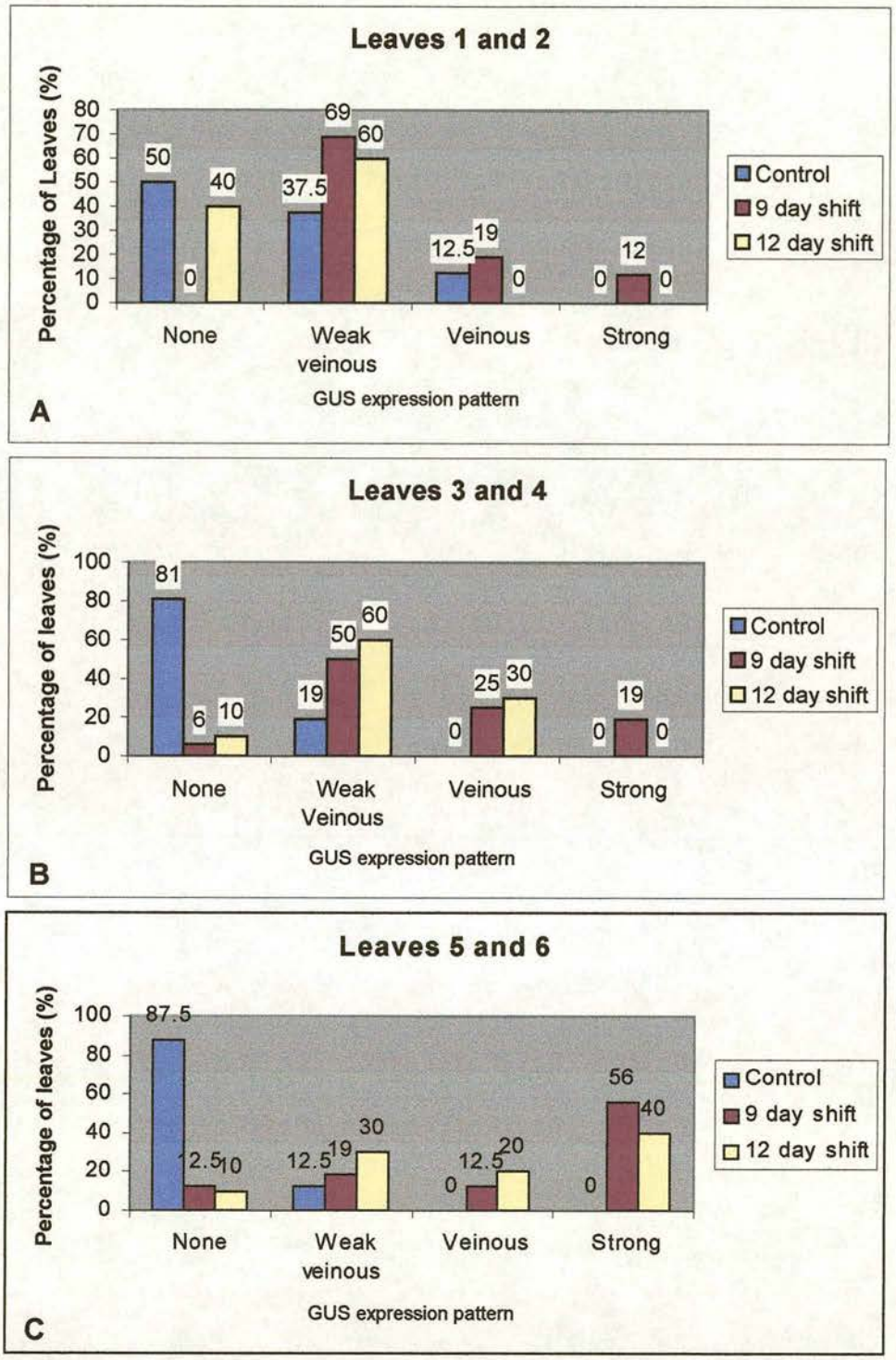
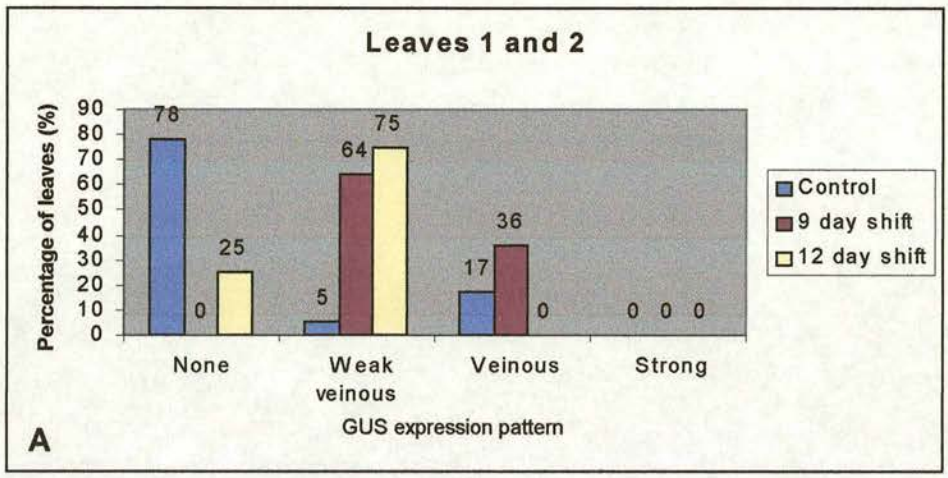
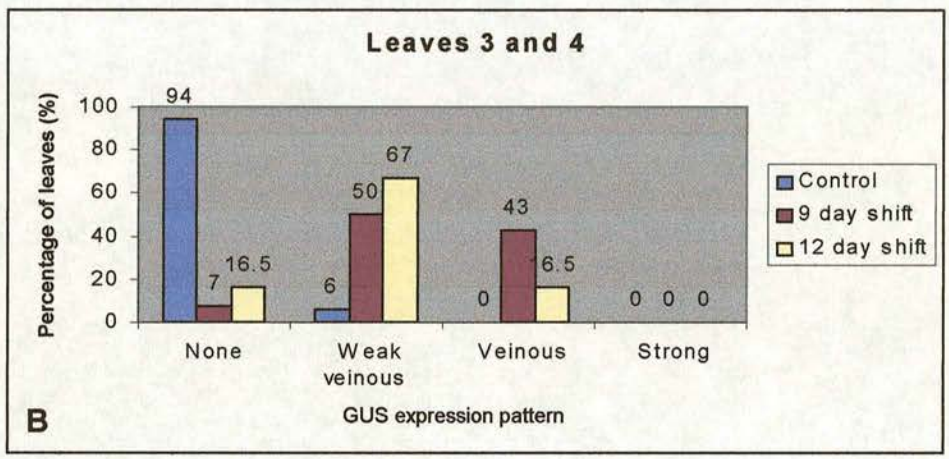


Figure 4.8 GUS expression in the leaves of K57 plants shifted off DEX compared to control plants, at S+20 (9-day shift) and S+17 (12-day shift). GUS activity was analysed in the first six leaves of plants shifted at 9 days (n = 8) and 12 days (n = 5). Control plants (n = 8) were grown in the presence of DEX from germination and analysed on the same day. All plants were 29 days old at the time of analysis.





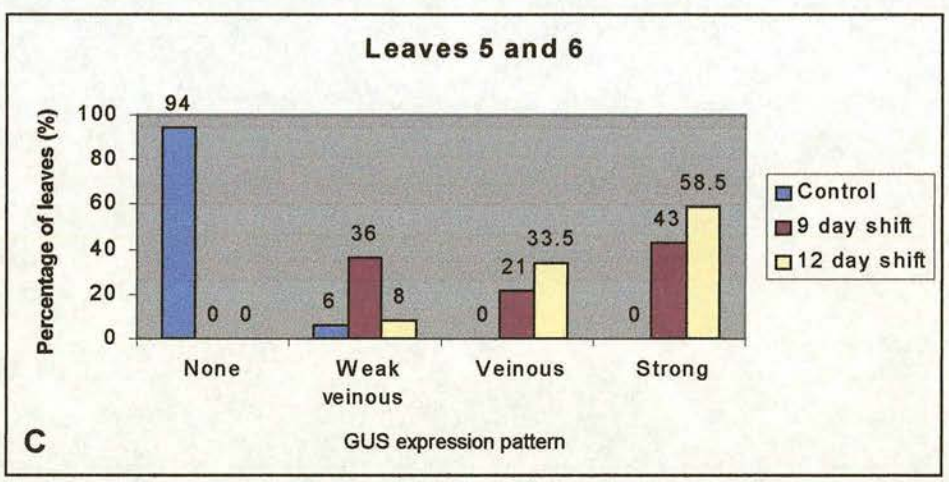


Figure 4.9 GUS expression in the leaves of K57 plants shifted off DEX compared to control plants at S+24 (9-day shift) and S+21 (12-day shift). GUS activity was analysed in the first six leaves of plants shifted at 9 days (n = 7) and 12 days (n = 6). Control plants (n = 9) were grown in the presence of DEX from germination and analysed on the same day. All plants were 33 days old at the time of analysis.

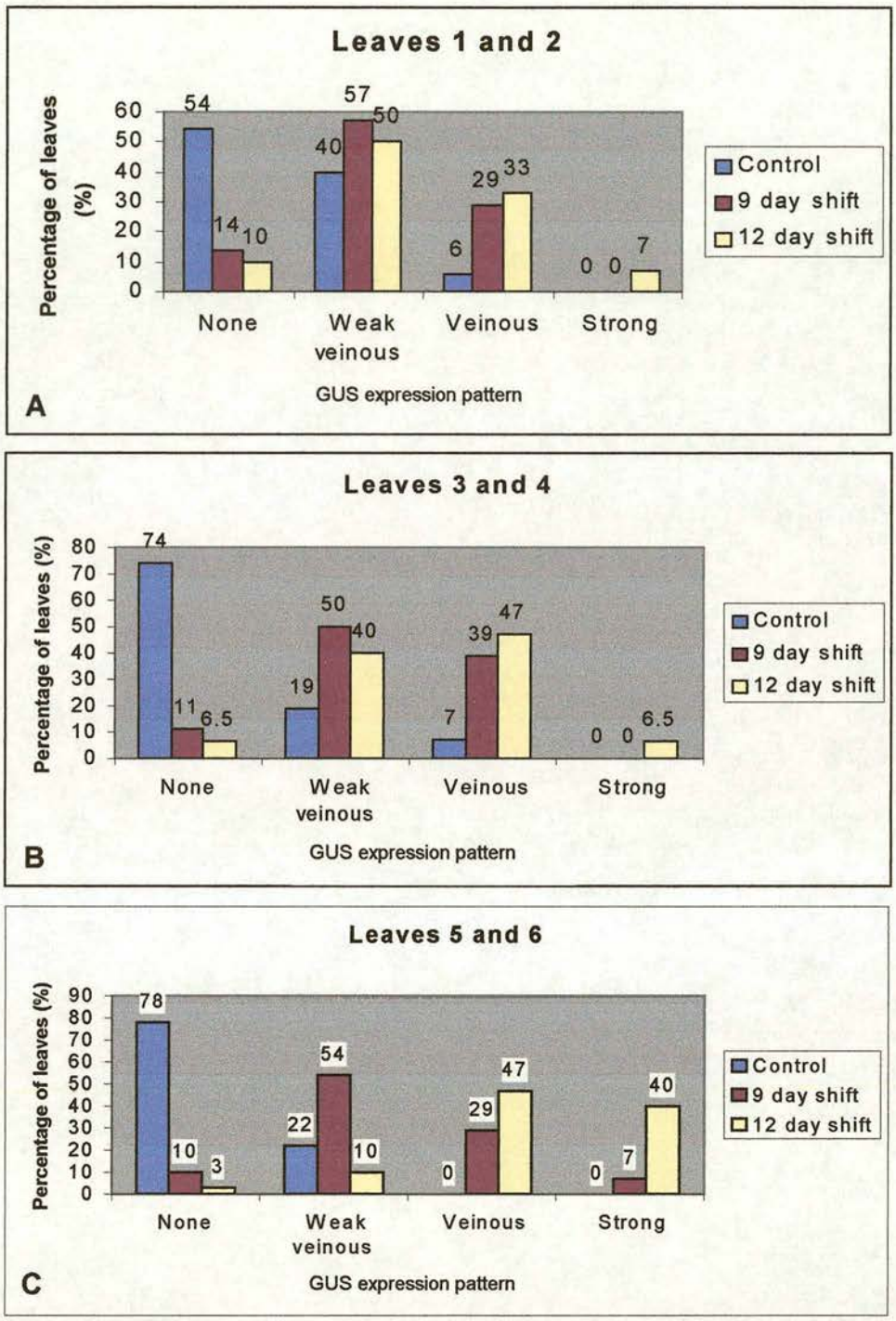


Figure 4.10 GUS expression in the leaves of K57 plants shifted off DEX compared to control plants at S+31 (9-day shift) and S+28 (12-day shift). GUS activity was analysed in the first six leaves of plants shifted at 9 days (n = 14) and 12 days (n = 15). Control plants (n = 34) were grown in the presence of DEX from germination and analysed on the same day. All plants were 40 days old at the time of the analysis

expression was not detected by RT-PCR in DEX treated seedlings at 14 days, and only a very low level of *AG* was detected in the cotyledon samples (Figure 4.3).

GUS analysis from nine day-shifted seedlings confirmed that *CLF* is required persistently to repress *AG* during leaf development (Figure 4.11). Strong *AG*-GUS expression is present in all leaves, including the first pair of leaves from shifted seedlings, 17 days after shifting (Figure 4.11c). In contrast no GUS activity is detected in control plants (Figure 4.11b). Figure 4.7 shows that the strongest GUS activity predominates in leaves 5 and 6 at this time. At later time points (S+31) GUS activity is present in the leaves (Figure 4.11e) and inflorescence stem (Figure 4.11g; Table 4.3a) of shifted plants, but not the control (Figure 4.11d and f; Table 4.3a). Curling of late rosette and cauline leaves in shifted plants at S+31 (Figure 4.12b and c) demonstrates the morphological effect of inactivating *CLF* during leaf development. The shift did not reduce the size of the rosette leaves although the curled cauline leaves were smaller than the cauline leaves of DEX grown controls (Figure 4.12c). GUS activity reveals strong *AG* misexpression in the stem of shifted plants at S+31 (Figure 4.11g). This does not affect the elongation of the stem since the height of the shifted and control stems are comparable (Figure 4.12b).

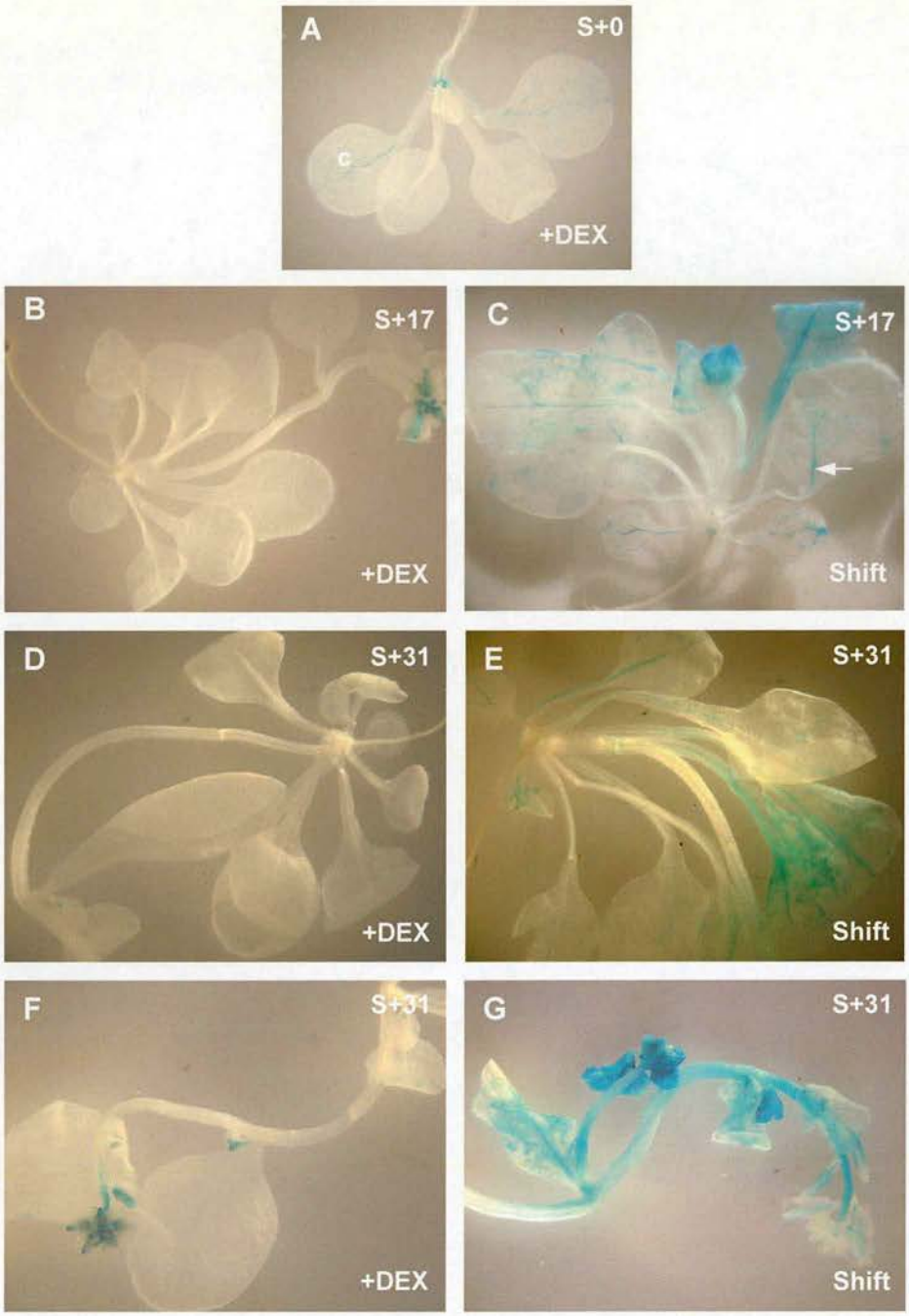


Figure 4.11 *AG*-GUS expression in K57 plants shifted off DEX at 9 days. Seeds of the K57 line were germinated and grown on plates treated with DEX. At day 9 half of the seedlings were shifted onto plates containing normal media (Shift). Control seedlings were treated with DEX throughout (+DEX). Whole seedlings were stained for GUS activity with X-gluc. At 9 days (S+0) *AG*-GUS expression is only present in the cotyledons **c** (A). 17 days after the shift (S+17) GUS expression is present in all the leaves of a shifted plant, including a veinous stain in the first pair of leaves (arrow, C) but absent from leaves of DEX grown plants (B). 31 days after the shift (S+31) GUS expression is present in the leaves (E) and stem (G) of shifted plants, but absent from the leaves (D) and stem (F) of DEX grown plants.

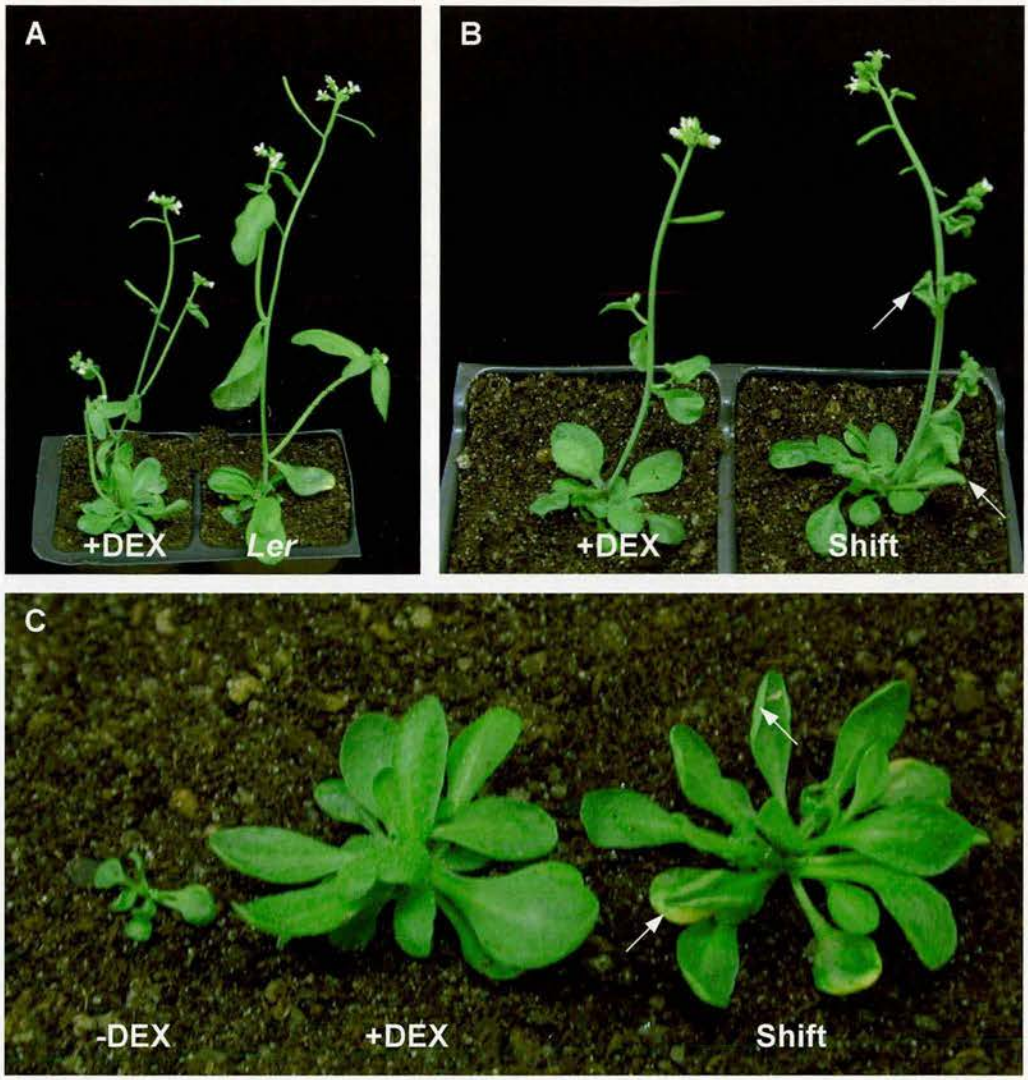


Figure 4.12 Leaf curling in K57 plants shifted off DEX at 9 days. K57 seedlings were germinated and grown on plates containing DEX. At day 9 seedlings were shifted to normal plates and later transferred to soil (Shift). All photographs were taken 31 days after the shift. Control plants were also grown on plates containing DEX and then transferred to soil where they were watered and sprayed with a DEX solution every other day (+DEX). (A) Control plants grown on DEX resemble *Ler* wild type plants except for a slight reduction in stem height and size of the cauline leaves. (B) Phenotype of plants shifted off DEX at day 9 (Shift) compared to controls grown on DEX throughout (+DEX). Curling is apparent in the late rosette and cauline leaves of shifted plants (arrows) indicating *AG* misexpression. Neither the expansion of the leaves or stem is affected by the shift. (C) Leaf curling is apparent in the rosette leaves of plants shifted off DEX (arrows), The shift has not reduced the size of the rosette leaves (centre and left), in comparison K57 plants grown without DEX (-DEX) have small rosette leaves.

Type of *AG* GUS expression in the stem. (%)		Days after shifting (day of shift = S+0)		
		S+20	S+24	S+31
9day shift seedlings	None	12.5	0	7
	Weak	0	14	7
	Strong	87.5	86	86
DEX grown controls	None	87.5	100	62
	Weak	12.5	0	32
	Strong	0	0	6

Table 4.3a) *AG* GUS expression in the stems of seedlings off DEX shifted at nine days

Type of *AG* GUS expression in the stem.		Days after shifting (day of shift = S+0)		
		S+17	S+21	S+28
12day shift seedlings	None	0	0	0
	Weak	60	33	0
	Strong	40	67	100
DEX grown controls	None	87.5	100	62
	Weak	12.5	0	32
	Strong	0	0	6

Table 4.3b *AG* GUS expression in the stems of seedlings shifted off DEX at 12 days.

To investigate whether *CLF* is required throughout leaf development to repress *AG* it was necessary to compare the first pair of control leaves to shifted leaves. The presence of leaky induction in the first set of leaves precludes a direct comparison. Despite this the results indicate greater misexpression in the first set of leaves as a result of shifting. *AG*-GUS misexpression is already present within 87% of these leaves by S+17 (Figure 4.7a), misexpression is 100% by S+20 (Figure 4.8a) only falling slightly to 86% at S+31 (Figure 4.10a). Misexpression in the majority of these leaves was veinous (Figure 4.11c and e) although strong expression was also present at earlier time points (Figure 4.7a and 4.8a).

To gain further evidence that *CLF* is required during the later stages of leaf development to repress *AG*, plants were also shifted at day 12. At 12 days seedlings were separated into two classes, the more advanced class had four rapidly expanding leaves, leaves 3 and 4 being of a similar size (Figure 4.13a), the less advanced class had three mature expanding leaves and a fourth that was much smaller. Analysis from the more advanced class has been presented here since leaves 3 and 4 were analysed as a pair. *AG-GUS* expression was monitored in the 3rd and 4th leaves to determine whether *CLF* is required to repress *AG* in leaves during the later expansion phase of their development. *AG* is reliably repressed in the 3rd and 4th leaves of continuously induced plants allowing for a more reliable comparison.

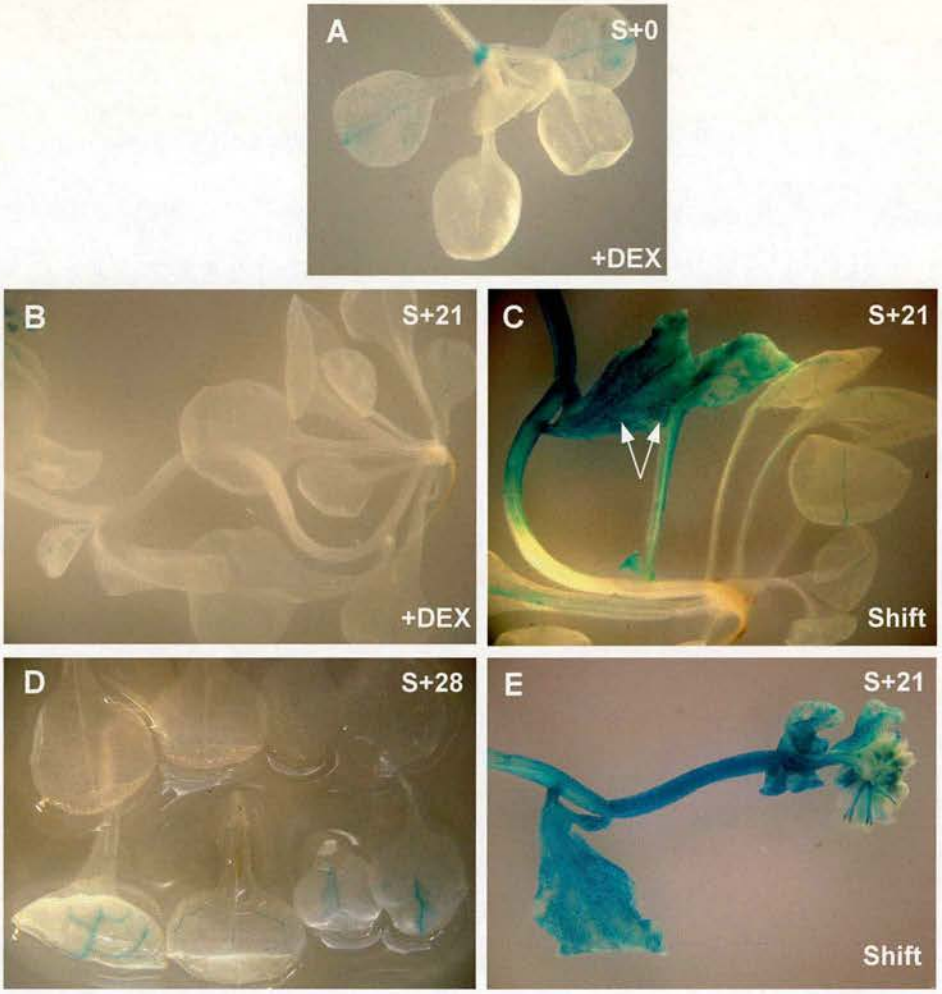


Figure 4.13 *AG*-GUS expression in K57 plants after a 12 day shift off DEX. Seeds of the K57 line were germinated and grown on plates treated with DEX. At day 12 half of the seedlings were shifted onto plates lacking DEX (Shift). Control seedlings were treated with DEX throughout (+DEX). Whole seedlings were stained for GUS activity with X-gluc. At 12 days (S+0) GUS expression is absent from the first four leaves and only present in the cotyledons (A). 21 days after the shift (S+21) GUS expression is present in the leaves of shifted plants (C) but absent from leaves of DEX grown plants (B). Strong GUS expression is present in the cauline leaves (C, arrows) and stem (E) of the shifted plant. Veinous GUS expression is present in the first four leaves of shifted plants (C, D). (D) Veinous GUS expression in leaves 1 to 4 (Bottom row, right to left) from a shifted plant (S+28), no GUS expression is present in leaves 1 to 4 from a DEX grown plant (Top row, right to left).

GUS expression reaches 90% in the 3rd and 4th leaves 17 days after the shift date (Figure 4.8b) and remains high at later shifts (Figure 4.9b and 4.10b). The expression in these leaves is veinous (Figure 4.13c and 4.13d). In contrast the majority of 3rd and 4th leaves from DEX controls at these times are free from GUS expression (Figure 4.8b, 4.9b and 4.10b; Figure 4.13b and d). These results are consistent with *CLF* being required continuously to repress *AG* during leaf development In common with the other shifting experiments strong *AG* GUS expression was present in younger leaves and the stem (Figure 4.13c and e; Table 4.3b).

4.4.2 Monitoring *AG* expression in the K57 line after restoring CLF+ activity

Seedlings germinated and grown on plates lacking DEX were transferred onto plates containing DEX at particular developmental times. These shifts were used to determine the requirement for CLF during early leaf development and also aimed to determine whether CLF could repress *AG* in tissues where *AG* had previously been misexpressed. In an initial experiment, *AG* expression was analysed by RT-PCR after I38 seedlings were shifted onto GM+DEX plates after 10 days on GM plates (Figure 4.14). The *AG* expression levels of shifted seedlings were compared to control seedlings, grown without DEX throughout. *AG* expression in shifted samples was comparable to control samples up to 72 hours after the shift, indicating that reactivated *CLF* was unable to reverse *AG* misexpression in the leaves.

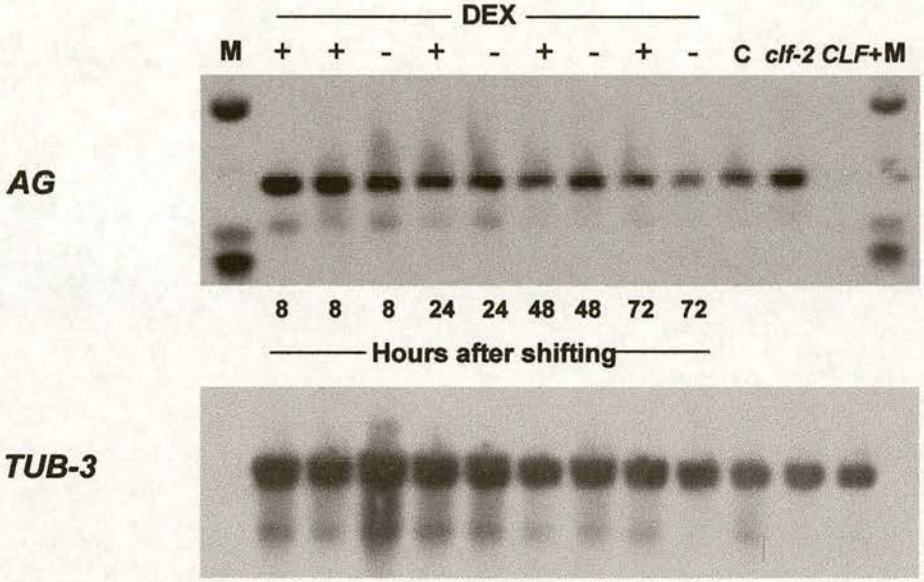


Figure 4.14 RT PCR analysis of *AG* expression in K57 seedlings after a shift onto DEX. K57 seedlings were transferred from normal media to DEX containing media (+DEX) at 10 days. During shifting each plant was dipped in a 10 µM solution of DEX (0.1% ethanol containing 0.001% silwet). *AG* expression was monitored in total RNA samples collected 8, 24, 48 and 72 hours after the shift. Control plants were left on normal media and analysed at the same timepoints as the shifts (-DEX). A separate control (C) was grown without DEX and transferred to a similar plate at the time of the shift, to mimic the shifting conditions these seedlings were also dipped in an 0.1% ethanol solution containing 0.001% silwet.β*TUBULIN-3 (TUB-3)* was used as an equal loading/amplification control.*clf-2* and *CLF+ (Ler)* were used to show differential *AG* expression. After sub maximal RT-PCR the PCR products were subjected to electrophoresis on agarose gels and then transferred onto Nylon membranes and probed with *AG* and *TUB-3* DIG probes. M=marker.

The initial shifting experiment, using the K57 line to analyse GUS activity, included a number of shifting time points to make an initial assessment. Shifts were carried out after growth in the absence of DEX for 5, 8, 12, 15 and 17 days. The results strongly indicated that CLF was unable to repress *AG* after it has been misexpressed in the leaves. For example the first pair of leaves of seedlings shifted at 5 days remained small and curled with strong *AG*-GUS expression up to 17 days after shifting. In contrast the 3 or 4 leaves had little or no *AG*-GUS expression and normal morphology (Figure 4.15c). A control was grown without DEX (Figure 4.15b). In an attempt to assess the developmental stage of each primordia at the time of the 5 day shift, 5 day samples were cleared and investigated under the microscope using Namarski optics, primordia 1 and 2 could be clearly seen but primordia 3 and 4 could not be clearly identified (Figure 4.15a).

A large scale shifting experiment was carried out using a 9 day shift. This was a convenient time to use since all seedlings had a pair of mature leaves by this time that already showed strong *AG*-GUS expression (Figure 4.20a). Monitoring the *AG*-GUS expression in these leaves and the next immature leaves would then show if reactivation of *CLF* could re-establish *AG* repression. *AG*-GUS expression was analysed on the day of the shift. The next analysis was carried out 17 days after the shift (S+17), when *AG* repression in young growth was predicted from the initial experiment. Three later time points (S+20, S+24 and S+31) were also analysed.

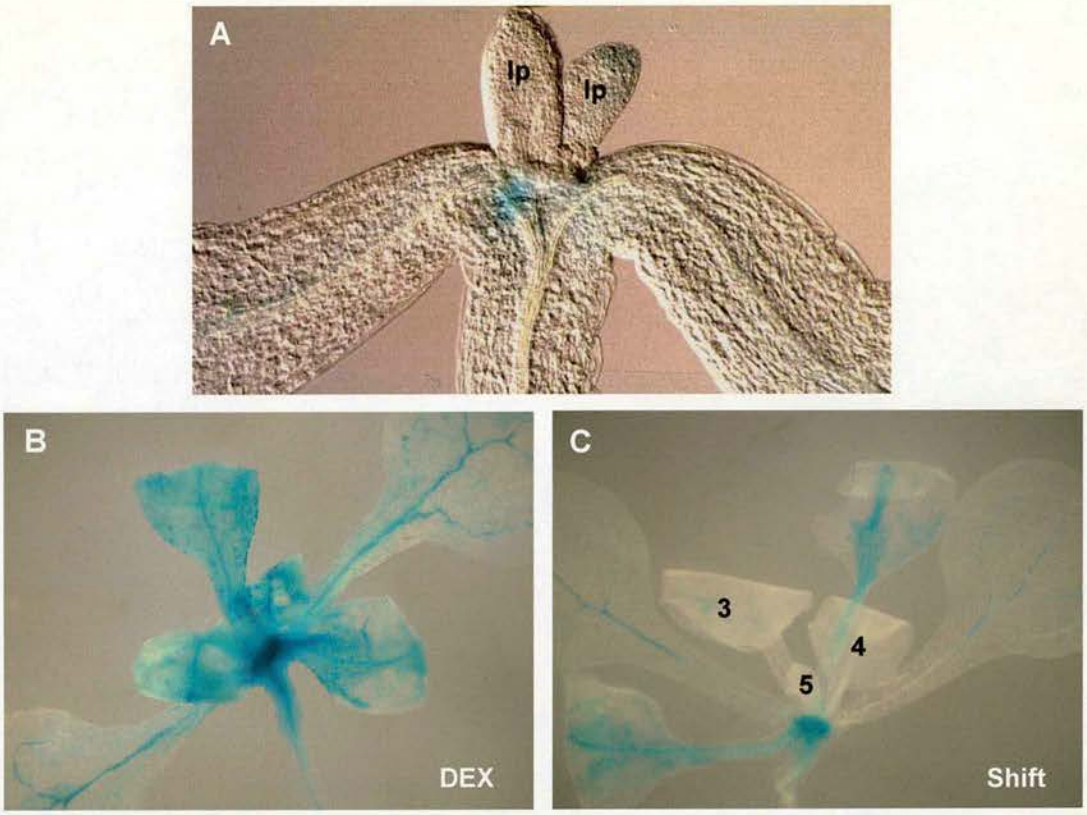


Figure 4.15 The effect of shifting K57 plants onto DEX at 5 days. (A) At five days a K57 seedling germinated and grown in the absence of DEX has two prominent leaf primordia (lp). The seedling was cleared using a chloral hydrate solution and viewed using Namarski optics. Seedlings were shifted at 5 days and monitored regularly. (C) Eight days after shifting *AG*-GUS expression is present in leaves 1 and 2 but absent from leaves 3,4 and 5 of a shifted plant (Shift). (B) AG-GUS expression is present in all the leaves of a control plant germinated and grown in the absence of DEX (-DEX) and analysed 8 days after the shift.

AG-GUS expression was not reduced or lost in the first four leaves of shifted plants as a result of shifting, demonstrating that unless *CLF* is supplied very early in leaf development it is unable to repress *AG* in leaves in which *AG* is already misexpressed (Figure 4.20; Figures 4.16-4.19). Strong *AG*-GUS expression persists in the first 4 leaves at the 24-day (S+24) (Figure 4.20b and d) and 31-days (Figure 4.19a and b) time points after shifting. Controls grown without DEX have strong *AG*-GUS expression in the first six leaves at S+17 (Figure 4.16). At later time points reduction in the intensity of GUS expression occurs (Figure 4.18 and 4.19). This indicates a normal decrease in *AG* misexpression in mature leaves in non-induced plants. This reduction seemed to be prevented in shifted plants so that their first four leaves showed even greater *AG*-GUS misexpression than control leaves at the later time points. Leaves 5 and 6 are also affected by the shift. At S+17 *AG*-GUS expression is present in over 90% of these leaves although only 8% had strong GUS expression (4.16c). At later time points the % of leaves with *AG*-GUS expression and the strength of the GUS activity decreases (Figure 4.17c, 4.18c and 4.19c) but never becomes equivalent to the DEX grown control.

In contrast to control seedlings (grown in the absence of DEX), plants shifted onto DEX at nine days had little or no *AG*-GUS expression in the stem (Table 4.4, Figure 4.20f). Shifting plants onto DEX at nine days also prevented the reduction in stem height present in non induced plants (Figure 4.21a). Figures 4.21b and c show the

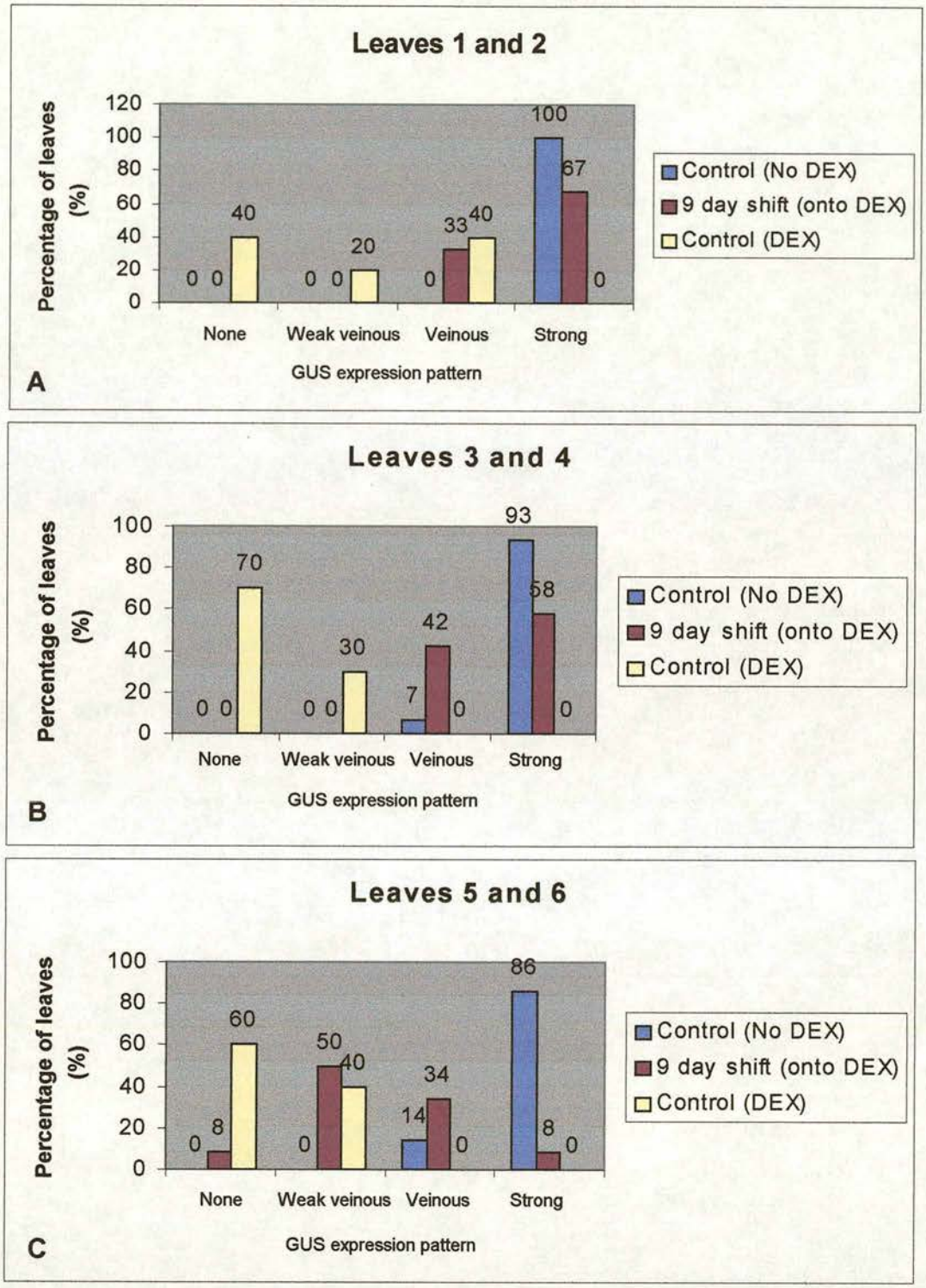


Figure 4.16 GUS expression in the leaves of K57 plants shifted onto DEX at 9 days compared to controls at S+17. GUS activity was analysed in the first six leaves of plants shifted onto DEX at 9 days (n = 6). The controls used were plants grown in the absence of DEX from germination (Control (No DEX); n = 7) and plants grown in the presence of DEX from germination (Control (DEX); n = 5). All plants were 26 days old at the time of analysis.

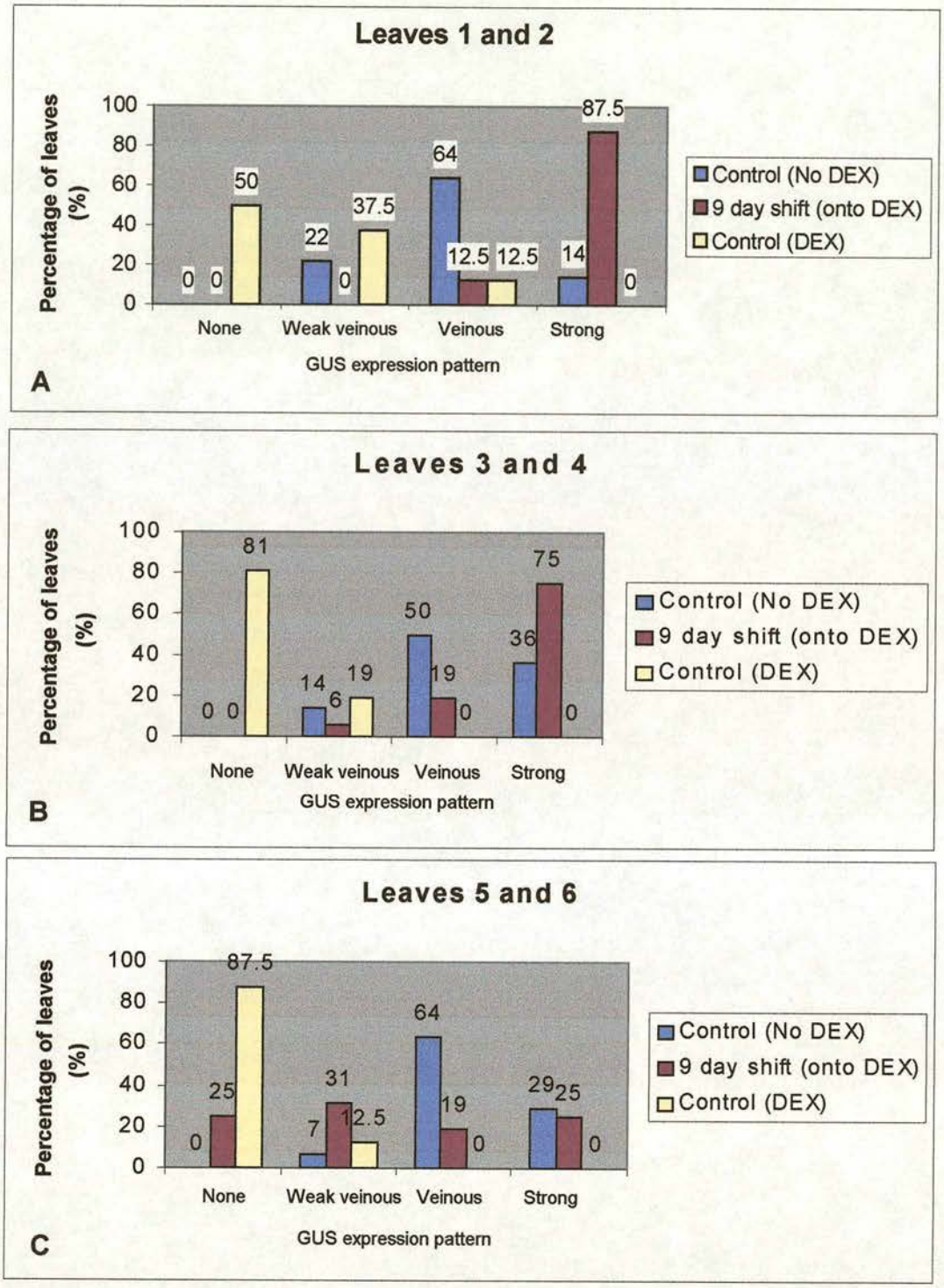


Figure 4.17 GUS expression in the leaves of K57 plants shifted onto DEX at 9 days compared to controls, 20 days after the shift (S+20). GUS activity was analysed in the first six leaves of plants shifted onto DEX at 9 days (n = 8). The controls used were plants grown in the absence of DEX from germination (Control (No DEX); n = 7) and plants grown in the presence of DEX from germination (Control (DEX); n = 8). All plants were 29 days old at the time of analysis

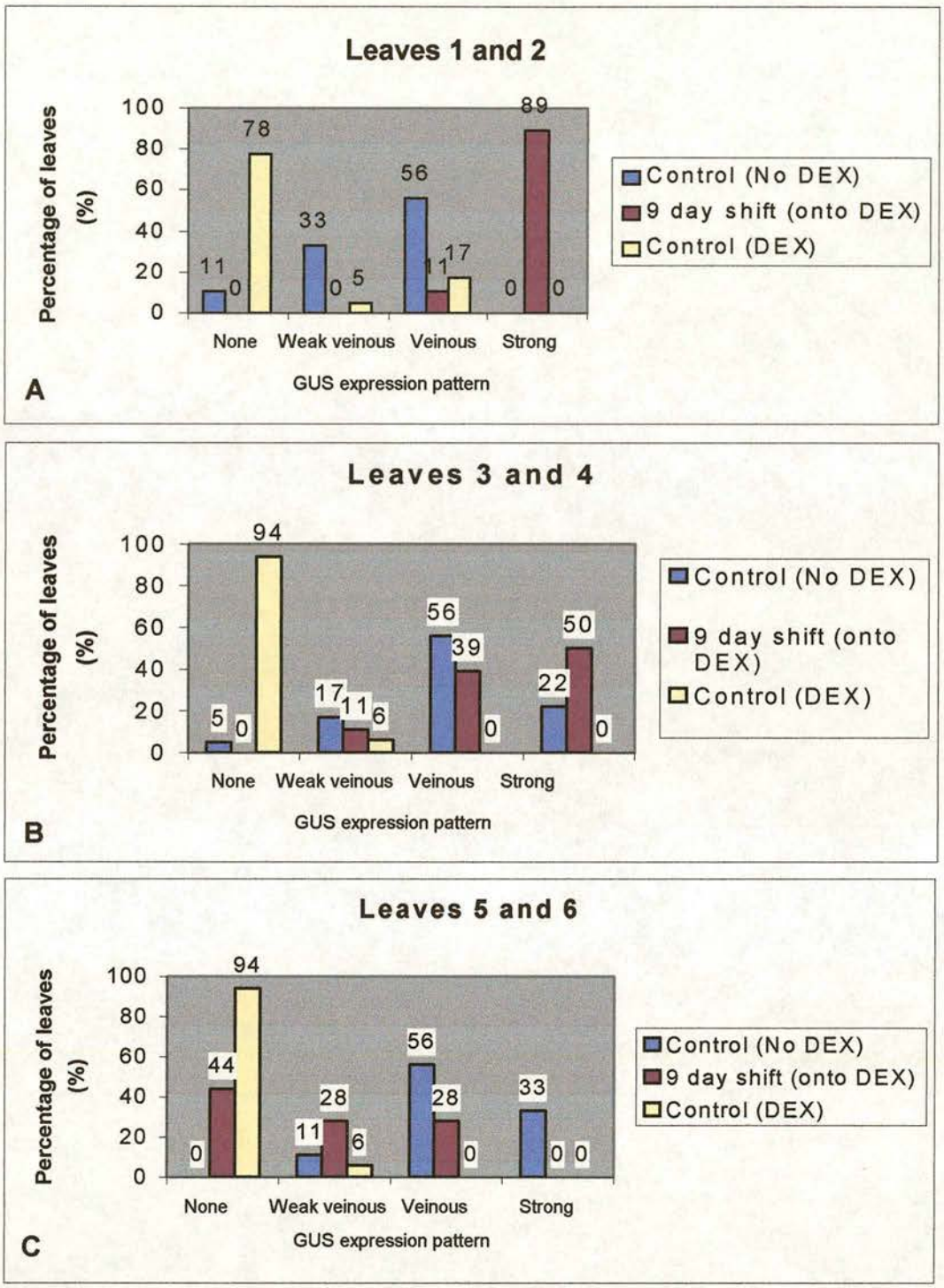


Figure 4.18 GUS expression in the leaves of K57 plants shifted onto DEX at 9 days compared to controls, 24 days after the shift (S+24). GUS activity was analysed in the first six leaves of plants shifted onto DEX at 9 days (n = 9). The controls used were plants grown in the absence of DEX from germination (Control (No DEX); n = 9) and plants grown in the presence of DEX from germination (Control (DEX); n = 9). All plants were 33 days old at the time of analysis.

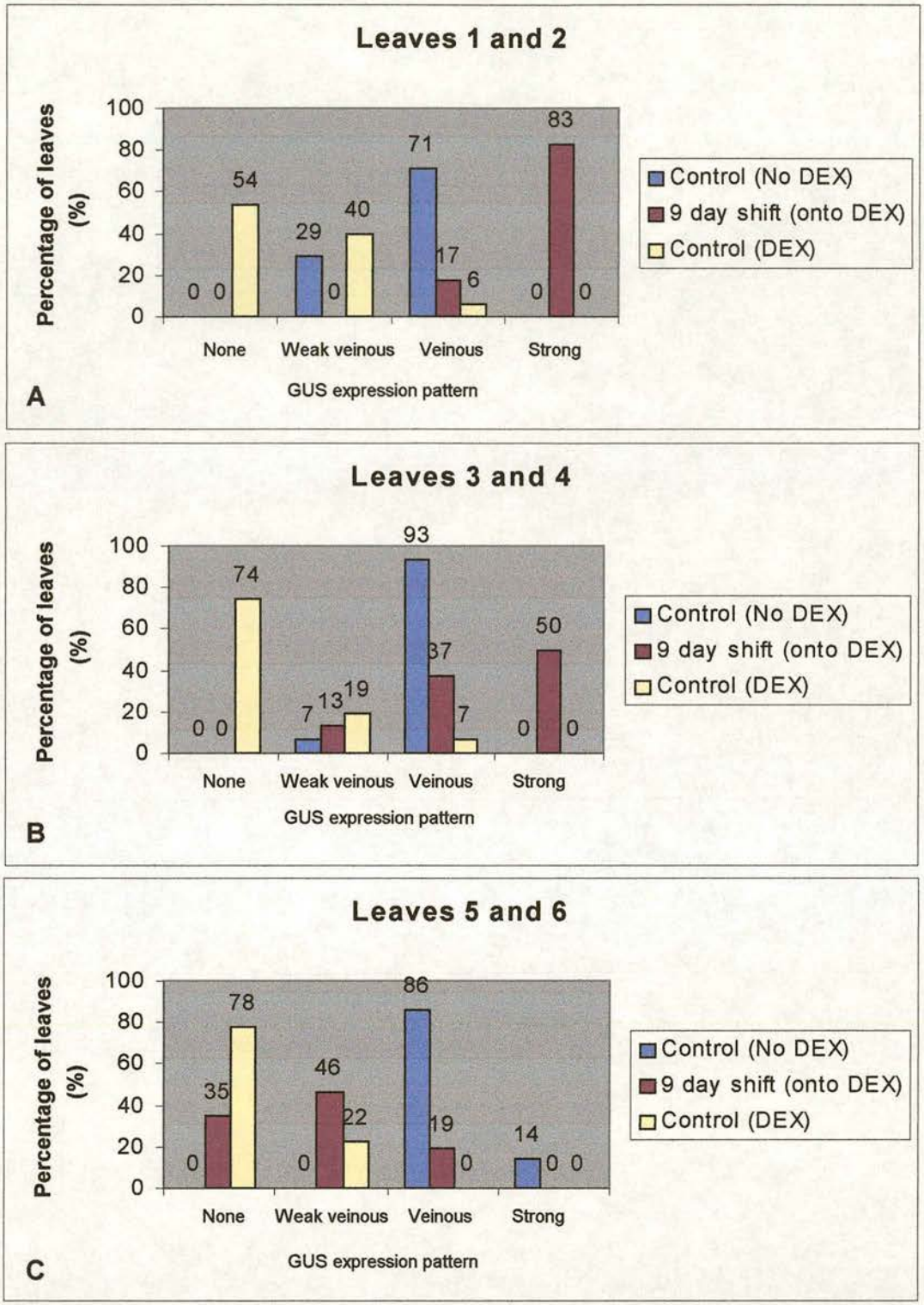


Figure 4.19 GUS expression in the leaves of K57 plants shifted onto DEX compared to controls, 31 days after the shift (S+31). GUS activity was analysed in the first six leaves of plants shifted onto DEX at 9 days (n = 23). The controls used were plants grown in the absence of DEX from germination (Control (No DEX); n = 7) and plants grown in the presence of DEX from germination (Control (DEX); n = 34). All plants were 40 days old at the time of analysis.

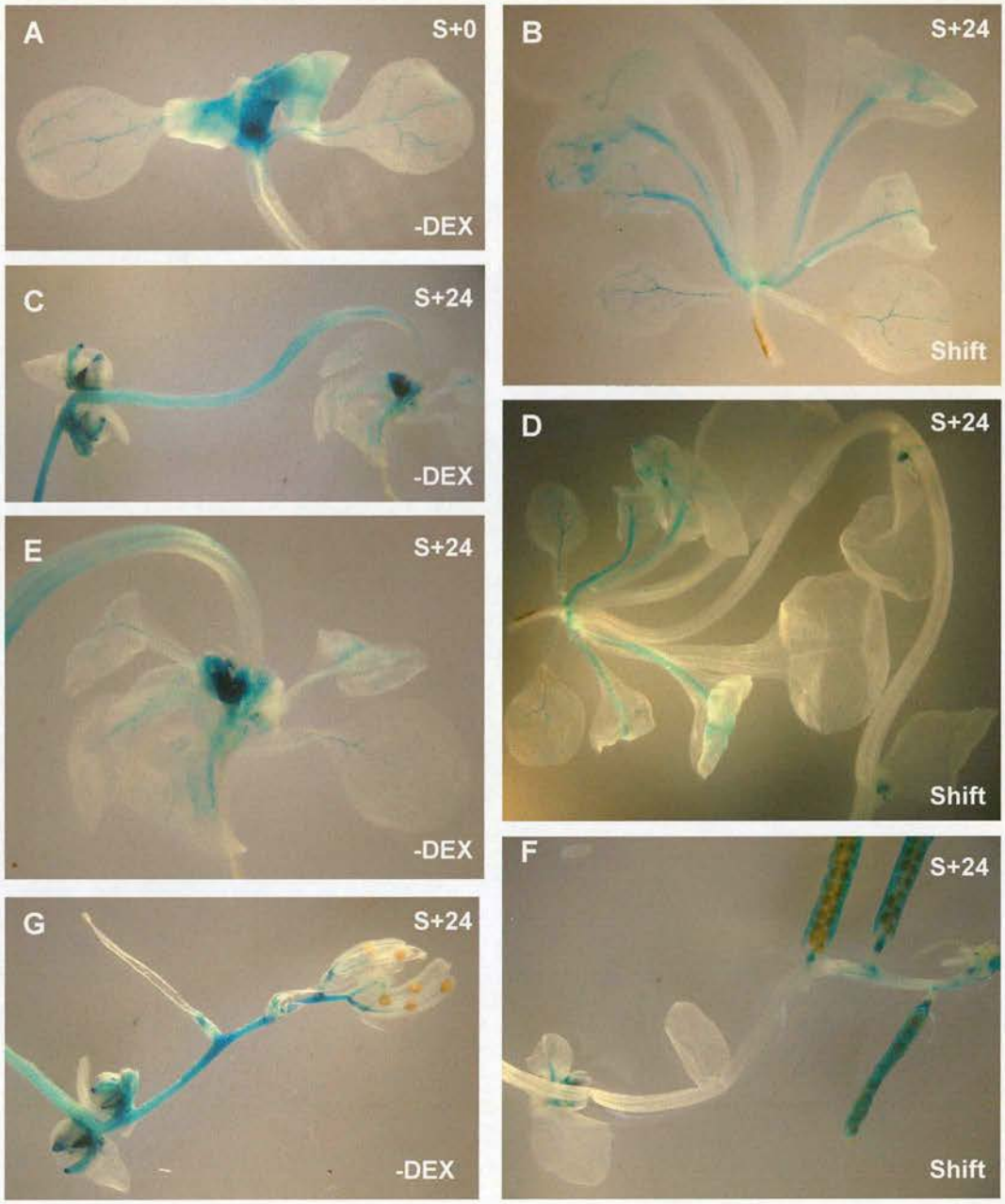


Figure 4.20 *AG*-GUS expression in K57 plants after shifting onto DEX at 9 days. Seeds of the K57 line were germinated and grown on plates in the absence of DEX. At day 9 half of the seedlings were shifted onto plates containing DEX (Shift). Control seedlings were grown continuously without DEX (-DEX). Whole seedlings were stained for GUS activity with X-gluc. At 9 days (S+0) strong GUS expression is present in the leaves (A). 24 days after the shift (S+24) strong GUS expression is still present in the first four to five rosette leaves (Leaves 1 to 5) of plants shifted onto DEX (B). Younger rosette leaves (Leaves 6, 7 etc); cauline leaves and the stem of the shifted plants (S+24) have no GUS expression (D, F). In contrast GUS expression is present in all of the leaves and the stem when plants are grown without DEX (C, E, G).

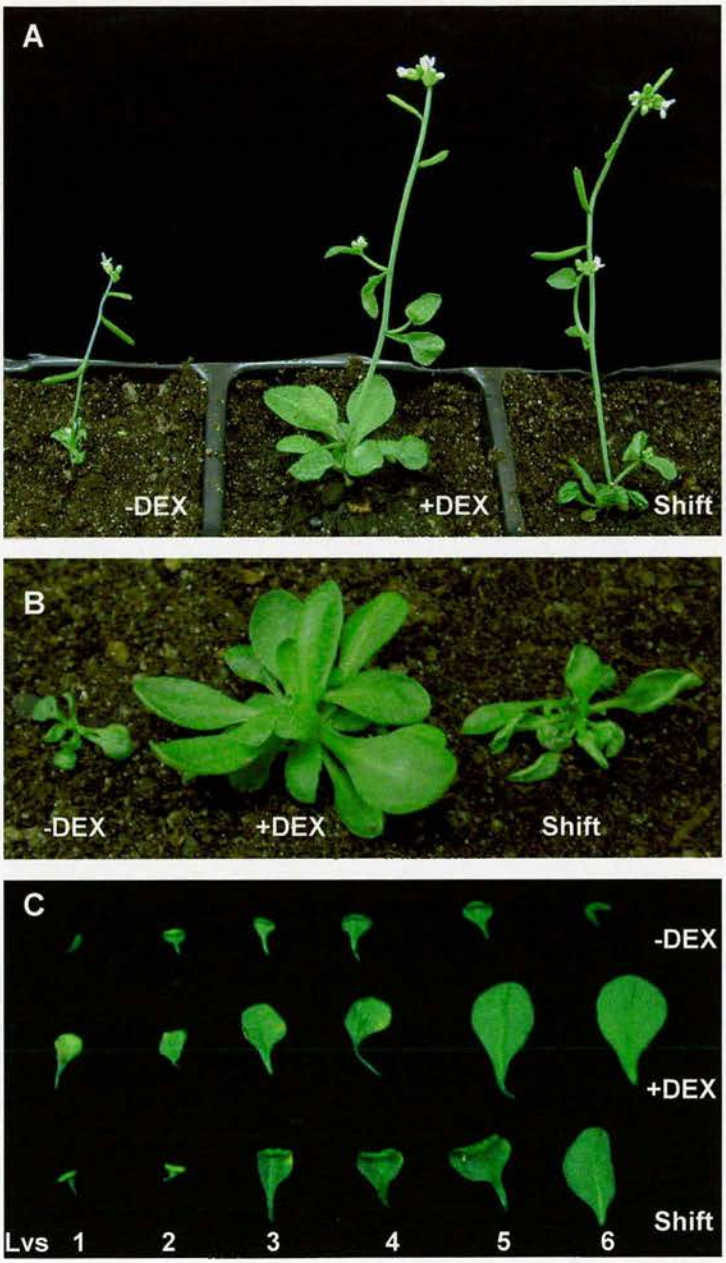


Figure 4.21 Phenotype of K57 plants shifted onto DEX at 9 days.
K57 seedlings were germinated and grown on plates in the absence of DEX. At day 9 seedlings were shifted onto plates containing DEX and later transferred to soil where they were watered and sprayed with a DEX solution every other day (Shift). All photographs were taken 31 days after the shift. Control plants were grown on DEX continuously (+DEX) or in the absence of DEX (-DEX). (A) Stem and cauline leaf development is rescued in plants shifted onto DEX. The shifted plants have a reduced rosette size (A, B). (C) Early rosette leaves (Leaves 1 and 2) of a shifted plant remain small and curled resembling the early leaves of a plant grown without DEX. Leaves 3 to 6 approach the size of DEX grown leaves (3 to 6), leaf curling also decreases between leaves 3 and 6 so that leaf 6 resembles a DEX grown leaf.

dramatic effect of the shift on the morphology of the rosette leaves. The oldest leaves are small and curled, resembling controls grown in the absence of DEX. Phenotypic rescue increases between leaves 3 to 6 indicating a reduction in *AG* misexpression.

Type of *AG* GUS expression in the stem (%).		Days after shifting (day of shift = S+0)			
		S+17	S+20	S+24	S+31
9day shift seedlings	None	100	87.5	66	74
	Weak	0	12.5	44	22
	Strong	0	0	0	4
Controls grown in absence of DEX	None	0	0	0	0
	Weak	14	0	0	0
	Strong	86	100	100	100

Table 4.4 *AG* GUS expression in the stems of seedlings shifted onto DEX at 9 days

4.5 Discussion

4.5.1 Introduction

A transgenic line conferring steroid inducible CLF+ activity was used to investigate the developmental time of CLF action. This represents the first time that a plant repressor has been studied using a steroid inducible system, since previous studies have involved transcriptional activators (Simon *et al*, 1996; Wagner *et al*, 1999; Samach *et al*, 2000). Transgenic *clf-2* mutant plants containing a gene fusion, *p(CLF)-CLF*::GR, encoding an in frame fusion of *CLF* and the glucocorticoid receptor under control of the *CLF* promoter were constructed by J. Goodrich. When plants containing the construct were grown in the presence of the steroid dexamethasone (DEX), phenotypic rescue was observed indicating that the CLF::GR fusion protein was functional when steroid induced. The *AG* reporter construct, pAG-I::*GUS* was then introduced into the steroid inducible CLF+ line so that *AG* expression could be easily monitored both temporally and spatially. One *clf-2* mutant line was obtained which was homozygous for both transgenes and this was named K57.

The pAG-I::*GUS* construct has previously been shown to faithfully replicate the expression pattern of the endogenous *AG* gene in wild-type plants (Sieburth and Meyerowitz, 1997). The pattern of expression in *clf-2* plants was also characterised

in this study, and this was further analysed in the present study. This confirmed that the pAG-I::*GUS* reporter contains *cis* acting regulatory sequences sufficient for its correct regulation by *CLF*. AG-GUS analysis of K57 plants grown with and without DEX revealed the expected differences in *AG*-GUS expression in the leaves. *AG*-GUS expression indicated misexpression of *AG* in plants grown in the absence of DEX, whilst the lack of *AG*-GUS expression in leaves of DEX grown plants demonstrated effective induction and activity of the CLF::GR fusion protein. Unexpectedly however, in cotyledons veinous *AG*-GUS expression was detected in plants grown both with or without DEX. One possible explanation was that the failure to silence AG::GUS in plants germinated with steroid was because steroid had not been supplied during seed development, when the cotyledons are formed. However this is unlikely because in a later experiment cotyledon *AG*-GUS expression could not be prevented even by constant treatment with DEX, including application during seed development. Steroid induction has also been shown to be incomplete during seed development in experiments using a *SHOOT MERISTEMLESS (STM)* steroid inducible construct (R.Sablowski personal communication). It may be that steroids cannot enter the embryo during seed development, or alternatively GR fusions do not respond to steroids during seed development and desiccation.

Two types of shifting experiments were carried out to determine the temporal requirement for *CLF*, the experiments involved shifting plants of the K57 line from DEX-containing to DEX-free media or *vice versa*. Lloyd *et al* (1994) used a similar

approach to define the pattern and timing of trichome development using a fusion between the maize regulatory factor R and GR. In the first type of shift, plants of the K57 line were grown in the presence of DEX so that the CLF::GR fusion protein was functional, and then shifted off DEX at particular development times, thus inactivating the CLF::GR fusion protein. These experiments aimed to discover whether *CLF* was required persistently during leaf development to repress *AG*. The second type of shift involved growing K57 plants without DEX, so that there was no functional CLF in the plants from the earliest stages of vegetative development, and then shifting onto DEX at particular developmental times thus activating the CLF::GR fusion protein. In these cases, *AG* will be misexpressed in leaves developing before the shift, therefore the experiments aimed to determine whether CLF+ activity can establish repression of *AG* in leaves where *AG* has previously been activated.

The results presented in this chapter demonstrate that *CLF* is required persistently to repress *AG* during leaf and inflorescence stem development. In addition, the results show that in leaves where *AG* is activated, restoring CLF+ activity is insufficient to repress *AG* unless supplied at very early stages of leaf development.

4.5.2 CLF is required persistently during leaf development

An initial shifting experiment carried out by J. Goodrich had suggested that *CLF* was required persistently during leaf development. Transgenic *clf-2* mutant steroid inducible CLF+ lines were grown in the presence of DEX for 17 days before being transferred onto a media lacking DEX. Leaf curling was observed in some leaves 13 days later, suggesting that *AG* was de-repressed in these leaves as a result of the shift. Using the K57 line, *AG*-GUS expression was monitored after similar shifting experiments. Preliminary shifts, using various developmental time-points, indicated that the curling of leaves previously observed had resulted from *AG* misexpression in the leaves. This misexpression only occurred a number of days after the shift. The long timescale probably reflects a number of factors, including persistence of DEX in plants and time taken for de-repression and GUS expression.

It is likely that DEX persists in the plant for some time after the shift so that CLF::GR is not immediately inactivated once plants are removed from DEX containing medium. In addition DEX is active at much lower concentrations (0.1μM, Aoyama and Chua personal communication) than those supplied (10μM). Some indications of the persistence of DEX in plants derives from the study of Aoyama and Chua (1997) who observed the induction level of DEX after transgenic steroid inducible tobacco plants were induced for two days with 30 μM DEX and then

transferred back onto media lacking DEX. The reporter used in this study was Luciferase, which has a short half-life. This reporter was used to monitor levels of DEX induction after plants were transferred off DEX. The authors found that levels of DEX induction began to decrease slowly for 3 to 4 days and then rapidly for the next 3 to 4 days. The timescale for persistence of DEX was not calculated in the experiments described here but is likely to have persisted for a shorter time than the study outlined above since the concentration of DEX used was 10 μM instead of 30 μM. In addition expression of the GR fusion in the Aoyama and Chua steroid inducible system is probably higher than the CLF::GR system, which is driven by the endogenous *CLF* promoter. In contrast Aoyama and Chua used 35S-GVG and also used a two component system which is likely to amplify the response.

A plant repressor has not previously been studied using a steroid inducible system. Rapid induction has been demonstrated in a number of studies involving steroid inducible plant activators (Simon *et al*, 1996;Wagner *et al*, 1999; Samach *et al*, 2000). In these cases induction was observed, between a few hours to a day, by activator induced transcription of downstream target genes. In these cases the shift was from a DEX-free media to a DEX-containing media facilitated a rapid change in the activity of the GR fusion proteins. In contrast looking for de-repression of a target of a repressor, using the opposite shift (DEX-containing to DEX-free), involves a delay caused by the time taken for DEX to decay to ineffective levels.

In the preliminary shifts using K57, *AG*-GUS expression was detected in all leaves from plants that had been shifted at 4 days, 6 days, 9 days and 12 days. This suggested that inactivation of CLF resulted in *AG* misexpression in both young leaf primordia, in the mitotic phase of leaf development, and more mature leaves which will be in the later cell elongation and differentiation phase of growth at the time of the shift. Analysis of *AG* mRNA expression in *clf-2* mutants revealed *AG* expression in young leaf primordia, indicating that CLF is required early in leaf development to repress *AG* (Goodrich *et al*, 1997). In an attempt to clarify the developmental timescale in which *CLF* is required within each leaf, large scale shifting experiments were completed using two chosen time points, 9 days and 12 days post germination.

At 9 days the first pair of leaves from K57 plants grown on DEX appear slightly smaller than the cotyledons (Figure 4.11a). Previous studies of *Arabidopsis* seedling development would suggest that these leaves have entered the later, cell expansion phase of development. A phase gradient exists between the base and the tip of the leaf. At the base of the leaf, cells are still in the earlier mitotic phase whereas cells at the tip are expanding and differentiating (Bowman, 1994). Expansion will become predominant and continue in this first pair of leaves until 20 or more days after germination. Leaf primordia 3 and 4 can also be easily distinguished by eye at day 9, primordia 3 slightly in advance of primordia 4 (Figure 4.11a). By day 9-10 after germination *Arabidopsis* grown under long day conditions starts to produce floral meristems, indicating that all of the rosette leaves have been initiated by this stage

(Bradley *et al*, 1997). This would suggest that primordia 5 and 6 have also been initiated in K57 plants shifted at 9 days.

By the first post-shift time-point,17 days after a shift to media lacking DEX, *AG*-GUS expression was detected in all leaves from day 9 shifted plants. Earlier rosette leaves were characterized by a veinous pattern of expression whereas later rosette and cauline leaves had stronger expression throughout the leaf. This gradient of *AG* misexpression, least in the oldest leaves and greatest in the younger leaves may reflect an increased requirement for *AG* repression in less mature leaves which are still in the mitotic phase. Kim *et al* (1998) propose that *CLF* affects cell division at an earlier stage of leaf development, and also affects cell elongation throughout the development of leaf primordia. It is possible that after the earlier stages of leaf development CLF+ activity is only required at a lower level to repress *AG*.

The results of the 9 days shifts off DEX confirmed that CLF+ activity is required throughout most of leaf development. The evidence of GUS expression analysis was strengthened by morphological data obtained from plants 31 days after the 9 day shift. The late rosette and cauline leaves of shifted plants were strongly curled indicating *AG* misexpression, in addition the cauline leaves were reduced in size in contrast to DEX grown controls (Figure 4.12). Although the early rosette leaves do express AG::GUS late in development following shifts off DEX, morphological effects were not observed. A likely explanation for this discrepancy is that expression

of *AG* late in leaf development does not have much effect as most cell division and expansion has been completed.

Using the 9 day shift it was not possible to show conclusively that *CLF* is required in more mature leaves undergoing expansion and differentiation. This was because silencing of AG::GUS in the first pair of leaves from DEX grown control plants was incomplete, for example >50% of these leaves displayed weak veinous or veinous *AG*-GUS expression at 26 days post germination (corresponding to the S+17 post-shift time-point). This may have been due to the embryonic derivation of the first pair of *Arabidopsis* leaves (McConnell *et al*, 2001). Despite this, increased *AG*-GUS expression was observed in the first pair of leaves as a result of the 9 day shift, for example at the third time-point (S+24) 100% of the first pair of leaves from shifted plants showed *AG*-GUS expression in comparison to 22% of the first pair of leaves from DEX grown control plants (Figure 4.9a). In addition persistence of DEX in plants after the shift means that it is likely that each leaf was probably at a more advanced stage of development when the CLF::GR protein fusion actually became non-functional than was indicated in the 9 day zero samples. Thus leaves 3 and 4 may have reached the later expansion phase of development by this time.

In order to determine whether *CLF* is required during the later stages of leaf development shifts were also carried out at 12 days. Figure 4.13a shows that at 12 days K57 plants grown on DEX have a mature first pair of leaves and two more leaves that are easily visible, leaves 3 and 4, each approximately half the size of

these. Observing whether *AG*-GUS expression occurs in leaves 3 and 4, which will have entered the later expansion phase of leaf development at the time of shifting, was used to investigate a late requirement for CLF. Comparison of *AG*-GUS expression in leaves 3 and 4 from shifted and DEX grown control plants could be made more confidently since silencing of AG::GUS in leaves 3 and 4 of DEX grown control plants was convincing. *AG* was misexpressed in the majority (over 80%) of leaves 3 and 4 from shifted K57 plants from the second to the last time point (S+17 to S+28) confirming that *CLF* is required even at the later stages of leaf development. The misexpression was restricted to the vascular system indicating perhaps that a *CLF* is only required in the vasculature in mature leaves to repress *AG*. In contrast approximately 50% of leaves 5 and 6 from 12 day shifted plants, which lost CLF activity during earlier mitotic growth, displayed strong *AG*-GUS misexpression indicating a requirement for a high level of CLF activity.

CLF is expressed persistently and ubiquitously during development (Goodrich *et al*, 1997) and the data presented here shows that this expression is linked to a persistent requirement for CLF activity during leaf development to maintain repression of *AG*. The situation would appear to be different during floral development where despite continuous and ubiquitous expression, requirement is spatially and temporally restricted *i.e. AG* only becomes ectopically expressed at late stages (stage 9 onwards) in the petals of *clf-2* mutant flowers (Goodrich *et al*, 1997). This difference may be attributed to cofactors present during floral organ development which spatially and temporally restrict the activity of *CLF* in the flower but not the leaves. For example

AP2 affects *AG* expression in the outer floral whorls, but does not affect *AG* expression in leaves.

CLF is the homologue of the *Drosophila* Pc-G gene *Enhancer of zeste* (*E*(*z*)). A number of studies using temperature sensitive *E*(*z*) alleles have concluded that *E*(z) is also required throughout development in order to maintain the repression of homeotic gene targets (Jones and Gelbert, 1990; Phillips and Shearn, 1990; Shearn *et al*, 1978), for example LaJeunesse and Shearn (1996) found that late loss of *E(z)* function resulted in misexpression of *Ultrabithorax (Ubx).* However Beuchle *et al* (2001) found little difference in *Ubx* expression in imaginal disc cells up to 96 hours after clones of cells were made E(z) deficient using an Flp-mediated mitotic recombination system. Some cells near the presumptive wing margin did require E(z) persistently to silence *Ubx* but the majority of cells did not. The authors suggest that in *Drosophila, E(z)* may only be required during embryo development

Pc-G genes function by maintaining the repression of homeotic genes and other targets in cell lineages where they were silenced early in embryogenesis. Unlike transcription factors, in most cases Pc-G gene products do not bind DNA, but may be recruited by protein-protein interaction. Consistent with this Pc-G members are components of multimeric protein complexes. Currently, two distinct Pc-G complexes have been identified which are believed to form repressive chromatin structure (Jones *et al*, 1998; Sewalt *et al*, 1998; Ng *et al*, 2000). In *Drosophila* E(z) interacts directly with another Pc-G protein Extra sex combs (Esc) in a 600-kDa

complex which is believed to have crucial early function, initiating Pc-G silencing after early acting gap gene repressors (Ng *et al*, 2000; van Lohuizen, 1999, review). Purification of the E(z)/Esc complex revealed evidence that it functions by modifying chromatin structure. Both a component of a chromatin remodelling complex, a histone binding protein called p55, and also a histone deacetylase, RDP3, were identified as subunits of the E(z)/Esc complex (Tie *et al*, 2001). Another complex named PRC1 contains many mammalian Pc-G proteins including Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and Sex combs on midlegs (Scm) (Shao *et al*, 1999, DHI). This complex is believed to have a later maintenance function and although *E(z)* is not part of this complex, evidence suggests that it affects the chromatin binding and therefore possibly the function of a number of its component proteins. Using a temperature sensitive *E(z)* allele, Rastelli *et al* (1993) observed dissociation of Psc from most chromosomal sites within one hour after E(z)+ activity was removed. The polytene chromosomes of larval salivary glands which were associated with loss of E(z)+ and dissociation of Psc become decondensed suggesting loss of repressive chromatin structure. Similar dissociation of Ph and Pc, as a result of E(z)+ loss, has also been demonstrated (Platero *et al*, 1995). The results of this study suggest that the plant *E(z)* homologue *CLF* is also persistently required.

Silencing by Pc-G complexes is heritable, cell undergo many cycles of division over the required timescale of Pc-G function (Reviewed in Ringrose and Paro, 2000). The molecular basis of heritable silencing is not well understood but conceptually is

believed to involve a 'tag'which marks the repressed target gene, ensuring that silencing is faithfully propagated during DNA replication and cell division. This tag would be required during DNA replication and also mitosis when a number of Pc-G proteins have been shown to dissociate from chromatin (Buchenau *et al*, 1998) as it becomes highly condensed in metaphase.

Certain histone modifications including methylation have been proposed as possible heritable 'tags'. Methylation patterns are propagated to all daughter cells after cell division cycles because hemi-methylated DNA strands, formed after DNA replication, are recognised by a methyl transferase and methylated on the daughter strand. Recently evidence has emerged which indicates that methylated histones can recruit methyl transferase via HP1 binding. Interestingly the methyl transferase in question was encoded by *SUV39H1*, a gene which is involved in epigenetic silencing of DNA which shares common structural motifs with Pc-G members (Rea *et al*, 2000; Lachner *et al*, 2001; Bannister *et al*, 2001).

Another form of histone modification, deacetylation, is believed to play a role in Pc-G function, however it is not clear how this form of histone modification could form a heritable tag. There is no known mechanism whereby hemi-acetylated nucleosomes present in the daughter cells after cell division could become fully deacetylated. Van der Vlag and Otte (1999) have demonstrated that the activity of histone deacetylase (HDAC) proteins is required for the normal function of embryonic ectoderm development (EED), a mammalian *Esc* homologue. The opposite of deacetylation is

hyperacetylation which has been shown to be associated with the heritable activation of a *Drosophila* chromosomal element *Fab-7* which is usually repressed by Pc-G proteins (Cavalli and Paro, 1999). The authors used a transgene carrying the *Fab-7* PRE flanked by reporter genes under control of the transcriptional activator GAL4. They found that a pulse of activation by GAL4 during embryogenesis was able to release Pc-G silencing (Cavalli and Paro, 1998). This activation was mitotically inheritable and independent of GAL4, in addition the activation state was associated with hyperacetylation of the associated chromatin.

The heritable tag could be self maintaining and independent of Pc-G proteins, for example methylation patterns could form a heritable tag which would only need to be set up by Pc-G proteins and would then propagate in a heritable manner. Conversely Pc-G proteins may be required continuously to ensure its maintenance. The continuous requirement for CLF+ activity determined in this study is consistent with a heritable tag which is dependent rather than independent of the presence of the repressive PcG complex containing CLF. Recently Beuchle *et al* (2001) also studied the heritable nature of Pc-G silencing. Pc-G members were depleted by making FLP recombinase induced mitotic clones and later resupplied by inducing a heat-shock promoter Pc-G transgene. The authors investigated whether the heritable tag or mark can persist through cell divisions in the absence of Pc-G proteins. They found that the heritable tag could persist in the absence of the Pc-G genes *Psc* and *Suppressor of zeste 2 (Su(z)2)*, but only for a few cell generations after depletion of the Pc-G genes , within this timeframe (48 hours) resupply of Pc-G activity resulted in re-

establishment of repression, however longer depletions (72 hours) prevented re-establishment of the silenced state. For other Pc-G genes including *Pc* and *Scm* any derepression of targets prevented re-establishment of silencing after resupply of Pc-G gene. These experiments indicate that the heritable tag is mainly dependent, rather than independent of Pc-G proteins in agreement with the conclusions of this study. However it should be noted that this study also classified *E(z)* as a type of Pc-G gene which is not required continuously but only required during embryogenesis.

4.5.3 In leaves where *AG* is activated, restoring CLF+ activity is insufficient to repress *AG*

Temperature sensitive alleles have been instrumental in determining the temporal requirement of the *Drosophila* gene *E(z).* However it has not been possible to determine the effects of supplying *E(z)* later in development, after early absence, due to the embryonic lethality of maternal null offspring and the early pupal lethality of zygotic null offspring from *E(z)*+/- mothers (Jones and Gelbert, 1993; Jones and Gelbert, 1990; Shearn *et al*, 1978) . In the experiments described here it was possible to grow plants of the K57 line in the absence of DEX until particular developmental times and then shift onto a DEX-containing media. The effects of these shifts were analysed by RT-PCR, *AG*-GUS expression analysis and morphology. The results demonstrated that CLF+ activity is unable to repress *AG* in leaves where *AG* had become activated.

In the first experiment K57 plants were shifted onto DEX-containing media at day 10 when strong *AG* misexpression would have been present in 4 to 5 leaves. RT-PCR analysis was used to analyse the *AG* expression levels in seedlings from shifted and control plants (grown without DEX) at particular time points after the shift. There was no decrease in *AG* expression, as a result of shifting, up to 72 hours after the shift. DEX induction has been shown to have rapid effects on gene activation (Simon *et al*, 1996; Wagner *et al*, 1999; Samach *et al*, 2000) therefore establishment of *AG* repression, if mechanistically possible, might be expected to result in down regulation of *AG* expression within 72 hours of shifting.

It is interesting to note that *AG* has not yet been confirmed as a direct target of CLF, it is also possible that CLF acts as an activator of a separate gene which in turn represses *AG,* or acts as a repressor of an activator of *AG.* The *Drosophila E(z)* gene has been shown to act in the maintenance of transcriptional activity by trithorax-group (trx-G) genes at particular gene locus (LaJeunesse and Shearn, 1996), in addition heat inactivation of temperature sensitive E(z) proteins reduces the chromosomal binding of the trx-G protein trithorax (trx) (Kuzin *et al*, 1994). Steroid inducible systems have been used to determine whether the targets of plant transcriptional activators are direct or indirect, using a combination of steroid induction and translational inhibition by cycloheximide (Wagner *et al*, 1999; Sablowski and Meyerowitz, 1998; Samach *et al*, 2000). Samach *et al* (2000) demonstrated that *FLOWERING LOCUS T (FT)* was a direct target of CONSTANS (CO) by observing induction of *FT* transcription by CO::GR within 2 hours of DEX

application in the presence of the protein synthesis inhibitor cycloheximide. In contrast *LEAFY (LFY)* mRNA was not induced if cycloheximide was supplied with DEX suggesting that this is an indirect target of *CO i.e.* protein synthesis of downstream targets is required in order to activate *LFY*. It may not be possible to determine the direct targets of CLF using a similar method since *CLF* has a repressive rather than an activation function. Additionally the RT-PCR experiment described above and the experiments discussed below shows that *AG* transcription responses to CLF::GR induction are either very slow or nonexistent. The use of cycloheximide requires a fast response because the plants cannot be kept alive for long after the treatment.

To gain further evidence that *AG*-misexpression persists in leaves after a shift to CLF+ conditions, *AG*-GUS expression was monitored in plants up to 31 days after a 9 day shift onto DEX. At the time of the shift K57 seedlings had a first pair of highly curled leaves with strong *AG*-GUS misexpression, primordia 3 and 4 are also visible (Figure 4.20a). Since leaf development is not retarded in *clf-2* mutants it is also likely that leaf primordia 5 and 6 have been initiated by nine days (Bowman *et al*, 1994). Detailed analysis of *AG*-GUS expression in the first six leaves showed that this misexpression remains in the first four leaves up to 31 days after the shift (Figures 4.16a,b; 4.17a,b; 4.18a,b; 4.19a,b; Figure 4.20 b, d) . A possible limitation of such a shifting experiment involves the persistence of the β-Glucuronidase activity, this has been shown to have a half-life of approximately 50 hours in plant tissue (Jefferson *et al*, 1987), however this is unlikely to have affected the results presented in this study

because the first time point for analysis was 17 days after shifting and analysis was continued up to 31 days after the shift. In addition rapid cell division in the leaf during this time would dilute residual AG::GUS protein. It is also possible that the fusion of the N-terminal AG protein to GUS alters its stability, possibly reducing the ½ life since this fusion has been shown to become restricted to particular cell types late in stamen and ovule development.

To reinforce the results of *AG*-GUS expression the morphology of shifted plants was analysed after a nine day shift onto DEX, leaves 1 to 5 remain curled 31 days after shifting, which could result either from transient early *AG* expression or the continued presence of strong *AG* expression. The results of the *AG*-GUS experiments suggest the latter. These results demonstrate that within each leaf primordia there is a critical early period when CLF+ activity must be present to establish the repression of *AG* in the leaf.

The exact developmental leaf stage when CLF+ activity is required could not be ascertained in these experiments since the developmental stage of each plant at the time of shifting was slightly different. However, leaf primordia 5 and 6 were differentially affected by shifting, for example at the second time point (S+20) (Figures 4.17c) 25% of these leaves had strong *AG*-GUS expression, whereas another 25% did not have any *AG*-GUS expresssion. This suggests that some of the 5 or 6th leaf primordia were at an early enough stage in their development to respond to the induction of CLF+ activity. In addition, in the 5 day shifting experiment, leaves 3

and 4 responded to the induction of CLF+, suggesting that they had not reached a point of no return before CLF+ activity was induced. An attempt was made to determine the developmental stage of these primordia at the time of shifting by clearing a 5 day old seedling and viewing the whole mount preperation under Nomarski optics, unfortunately the 3rd and 4th primordia could not be visualised in this experiments. Future experiments could closely determine the stage at which CLF+ activity is required by similar observation of the developmental stage of seedlings at the time of shifting. Clearing methods or *in situ* hybridisation could be used. If *AG* expression could be studied in life plants non-destructively during shifting experiments, the exact stage of each leaf primordia at the time of shifting could be determined and the results of shifting on each primordia could be analysed in detail.

All of the *Drosophila* Pc-G members are supplied maternally and zygotically and required early and persistantly, but the Pc-G gene *Esc* is different since it is only required transiently and early. Struhl and Brower (1982) used temperature-shift experiments to show that *Esc* is required during germ-band extension for about 6 hours, using a heat shock inducible system. Simon *et al* (1995) also demonstrated a critical window of requirement for *Esc* beginning between 2 and 4 hours of embryogenesis.

Two plant Pc-G genes of the *Fertilisation Independent Seed Development (FIS)* class are also required during an early phenocritical stage. Both *FERTILISATION-*

INDEPENDENT ENDOSPERM (FIE), the *Arabidopsis Esc* homologue, and *MEDEA (MEA),* an *Arabidopsis E(z)* homologue, are members of this class (Chaudury *et al*, 1997; Grossniklaus, 1998; Kiyosue *et al*, 1999; Ohad *et al*, 1996; Ohad *et al*, 1999). Vielle-Calzada *et al* (2000) have shown that many *Arabidopsis* genes that are active in the early embryo/endosperm do not have a critical early requirement. The authors found that many of these genes had a paternally silenced allele during early stages of embryogenesis and that even in the absence of maternally supplied product, late wild-type paternal product could still rescue the embryo. The *FIS* genes, in contrast, are required during early stages since wild-type paternal product is not sufficient to prevent embryo lethality if maternal product is not supplied (Grossniklaus, 1998). It is possible that FIS genes act by repressing specific targets to suppress cellular proliferation in the endosperm. The critical early requirement for these genes would be consistent with a "missing the boat" mechanism *i.e.* if FIS targets are switched on, FIS products are then unable to repress these targets. The results of the present study would suggest a similar mechanism. Beuche *et al* (2001) demonstrated that the Pc-G proteins Su(z)2 and Psc could re-establish silencing if re-supplied within 48 hours of clone induction (Pc-G depletion) but not after this time. In the case of Polycomb (Pc) resilencing of de-repressed homeotic genes was only possible if the protein was resupplied before de-repression of these genes had become fully established.

There are some additional studies which suggests that after switching a Pc-G silenced target to an active state this target remains active. Cavalli and Paro (1998)

used a transgene carrying the *Fab-7* PRE flanked by reporter genes under control of the transcriptional activator GAL4. They found that a pulse of activation by GAL4 during embryogenesis was able to release Pc-G silencing (Cavalli and Paro, 1998). This activation was mitotically inheritable and independent of GAL4. In addition the activation state was associated with hyperacetylation of the associated chromatin (Cavalli and Paro, 1999) suggesting prehaps that this pulse had switched the target chromatin from a deacetylated to an acetylated state. Histone deacetylation is required for E(z)/Esc function and so might be involved in plant Pc-G silencing. Using the histone deacetylase inhibitor Trichostatin A it might be possible to determine whether deacetylation is part of plant Pc-G silencing. The acetylation state of plant Pc-G targets such as *AG* could then be monitored during shifting experiments.

In summary, it has been shown that in leaves where *AG* is activated, restoring CLF+ activity is insufficient to repress *AG*. This indicates an early essential requirement for CLF during leaf development. The critical stage before which CLF must be active in leaf primordial, to establish *AG* repression was not tightly delineated. However, a reduction in *AG* misexpression in late rosette leaves (5 and 6) which would have been initiated at 9 days suggests that the critcal stage was post-initiation and probably during early primordia development.

4.5.4 CLF activity is required to repress *AG* in the inflorescence stem

Development from the shoot apical meristem is characterized by three defined phases in *Arabidopsis* (Ratcliffe *et al*, 1999). During the vegetative phase (V) the shoot apical meristem generates leaf primordia which form a rosette of leaves, this stage usually lasts for up to 7 days after sowing under long day conditions. Between 8 and 9 days the shoot apical meristem switchs to the first-inflorescence stage (I_1), providing that environmental and flowering time cues promote this. This short phase consists of the production of 2 to 3 cauline leaves. During the second inflorescence stage (I_2), between 10 and 12 days, the primary shoot elongates (bolts) and generates floral meristems.

Both types of shift performed using K57 were carried out at 9 days which corresponds to the I1 phase and shifts involving removal of DEX where also carried out at 12 days which corresponds to the I2 phase of development. The influence of shifting on *AG* misexpression in the inflorescence meristem was investigated. After K57 plants were shifted off DEX at 9 days, strong *AG*-GUS expression was detected in the inflorescence stem (Table 4.3a), especially at the S+17 and S+21 time-points. In contrast little or no *AG*-GUS expression was detected in the inflorescence stem of K57 plants grown on DEX throughout. Similarly, if K57 plants were shifted off DEX at 12 days, strong *AG*-GUS expression became evident in the inflorescence stem. This took longer to appear, at S+17 only 40% of plants had strong expression in the

stem. In contrast by S+28 100% of K57 shifted plants had strong *AG*-GUS expression in the inflorescence stem (Table 4.3b). These results show that CLF+ activity is required to prevent *AG* misexpression occurring in the inflorescence stem during both I1 and the beginning of I2.

In contrast no *AG*-GUS expression was detected in the inflorescence stem of K57 plants shifted onto DEX at 9 days. At S+17 and S+20 over 85% of these stems were free from any *AG*-GUS staining (Table 4.4). In contrast K57 plants grown in the absence of DEX throughout were characterised by strong inflorescence stem *AG* misexpression. This result indicates that CLF+ activity is not required before day 9 to prevent *AG* misexpression in the inflorescence stem. Thus, in combination, the analysis of shifting experiments indicates that CLF+ activity must be present at a particular phase of the I1, at about day 10 or 11 in order to prevent *AG* misexpression occurring in the inflorescence stem, which will begin to bolt at the beginning of the I2 phase (Ratcliffe *et al*, 1999).

It is interesting to note that the expansion of the inflorescence stem was not related to the presence or absence of *AG* expression. In both type of shift K57 plants produced inflorescence stems of a normal height, even though *AG* misexpression was present in one case and absent in another. The *clf-2* mutant is characterised by a reduced inflorescence height resulting from reduction in stem internode elongation. CLF+ activity until by 9, or alternatively after day 9 was sufficient to ensure normal inflorescence stem elongation.

4.5.5 Final conclusions

The results of this study indicate that CLF+ activity is required both at a critical early stage of leaf development and also persistently during leaf and inflorescence stem development to repress the *c* function floral homeotic gene *AG*.

References

Aalfs, J.D. and Kingston, R.E. (2000) What does chromatin remodelling mean? *Trends in Biochemistry.* **25**, 548-555.

Almeida, J., Rocheta, M. and Galego, L. (1997) Genetic control of flower shape in *Antirrhinum majus. Development.* **124**, 1387-1392.

Angell, S.M. and Baulcombe, D.C. (1999) Potato virus X amplicon-mediated silencing of nuclear genes. *The Plant Journal.* **20**, 357-362

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature.* **410**, 120-124.

Barlow, D.P. (1995) Gametic imprinting in mammals. *Science.* **270**, 1610-1613.

Bateson, W. (1894) *Materials for the study of variation treated with the especial regard to discontinuity in the origin of species,* pp. 84-85. MacMillan, New York and London.

Baulcombe, D.C. (1999) Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology.* **2**, 109-113.

Beuchle, D., Struhl, G. and Muller, J. (2001) Polycomb group proteins and heritable silencing of *Drosophila* Hox genes. *Development.* **128**, 993-1004.

Bowman, J.L. (1993) *Arabidopsis: an atlas of morphology and development.* New York, Springer-Verlag.

Bowman, J.L., Drews, G.N. and Meyerowitz, E.M. (1991a) Expression of the *Arabidopsis* floral homeotic gene *agamous* is restricted to specific cell types late in flower development. *Plant Cell.* **3**, 749-758.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989) Genes directing flower development in *Arabidopsis. Cell.* **1**, 37-52.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1991b) Genetic interactions among floral homeotic genes of *Arabidopsis. Development.* **112**, 1-20.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N. and Coen, E. (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of *Antirrhinum. Cell.* **72**, 85-95.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R. and Coen, E. (1997) Inflorescence commitment and architecture in *Arabidopsis. Science.* **275**, 80-83.

Bradley, D., Vincent, C., Carpenter, R. and Coen, E. (1996) Pathways for inflorescence and floral induction in *Antirrhinum. Development.* **122**, 1535-1544.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L. and Kassis, J.A. (1998) The *Drosophila* Polycomb group gene *pleiohomeotic* encodes a DNA binding protein with homology to the transcription factor YY1. *Molecular Cell.* **1**, 1057-1064.

Buchenau, P., Hodgson, J., Strutt, H. and Arndt-Jovin, D.J. (1998) The distribution of Polycomb-group proteins during cell division and development in *Drosophila* embryos: impact on models for silencing. *Journal of Cellular Biology.* **141**, 469-481.

Busch, M.A., Bomblies, K. and Weigel, D. (1999) Activation of a floral homeotic gene in *Arabidopsis*. *Science.* **285**, 585-587.

Busteria, A. and Bienz, M. (1993) A Polycomb response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *The EMBO Journal.* **12**, 1415-1425.

Busteria, A., Wightman, C.D. and Sakonju, S (1997) A silencer is required for maintenance of transcriptional repression throughout *Drosophila* development. *Development.* **124**, 4343-4350.

Carpenter, R. and Coen, E.S. (1990) Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus. Genes & Development.* **4**, 1483-1493.

Carpenter, R. and Coen, E.S. (1995) Transposon induced chimeras show that *floricaula,* a meristem identity gene, acts non-autonomously between cell layers. *Development.* **121**, 19-26.

Carpenter, R., Copsey, L., Vincent, C., Doyle, S., Magrath, R. and Coen, E. (1995) Control of flower development and phyllotaxy by meristem identity genes in *Antirrhinum. The Plant Cell.* **7**, 2001-2011.

Carrington, E.A. and Jones, R.S. (1996) The *Drosophila Enhancer of zeste* gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. *Development.* **122**, 4073-4083.

Chan, C.S., Rastelli, L., Pirrotta, V. (1994) A Polycomb response element in the *Ubx* gene that determines an epigenetically inherited state. *The EMBO Journal.* **13**, 2553-2564.

Chinwalla, V., Jane, E.P. and Harte, P.J. (1995) The *Drosophila* trithorax protein binds to specific chromosomal sites and is co-localized with *Polycomb* at many sites. *The EMBO Journal.* **14**, 2056-2065.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature.* **353**, 31-37.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G. and Carpenter, R. (1990) *flo*: a homeotic gene required for flower development in *Antirrhinum majus. Cell* **63**, 1311-1322.

Cavalli, G. and Paro, R. (1998) The *Drosophila fab-7* chromosomal element conveys epigenetic inheritance during mitosis and meiosis. *Cell* **93**, 505-518.

Cavalli, G. and Paro, R. (1999) Epigenetic inheritance of active chromatin after removal of the main *Trans* activator. *Science* **286**, 955-958.

Chaudury, A.M., Ming, L. Miller, C., Craig, S., Dennis, E.S. and Peacock, W.J. (1997) Fertilization-independent seed development in *Arabidopsis thaliana. Proceedings of the National Academy of Science (USA).* **94**, 4223-4228.

Chen, L., Cheng, J-C, Castle, L. and Sung, Z.R. (1997) *EMF* genes regulate *Arabidopsis* inflorescence development. *The Plant Cell* **9**, 2011-2024.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana. The Plant Journal* **16**, 735-743.

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1999) *PLENA* and *FARINELLI*: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. *The EMBO Journal* **18**, 4023-4034.

Devereaux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387-395.

Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell* **65**, 991-1002.

Eshed, Y., Baum, S.F. and Bowman, J.L. (1999) Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis. Cell.* **99**, 199-209.

Fauvarque, M.O. and Dura, J.M. (1993) polychromatic regulatory sequences induce developmental regulator-dependant variegation and targeted *P*-element insertions in *Drosophila. Genes and Development.* **7**, 1508-1520.

Fritch, C., Brown, J.L., Kassis, J.A. and Muller, J. (1999) The DNA-binding Polycomb group protein Pleiohomeotic mediates silencing of a *Drosophila* homeotic gene. *Development.* **126**, 3905-3913.

Gildea, J.J., Lopez, R. and Shearn, A. (2000) A screen for new trithorax group genes identified little imaginal discs, the *Drosophila melanogaster* homologue of human retinoblastoma binding protein 2. *Genetics.* **156**, 645-663.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M. and Coupland, G. (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis. Nature,* **386**, 44-51.

Grossniklaus, U., Spillane, C., Page, D.R. and Kohler, C. (2001) Genomic imprinting and seed development: endosperm formation with and without sex. *Current Opinion in Plant Biology,* **4**, 21-27.

Grossniklaus, U., Vielle-Calzada, J-P., Hoeppner, M.A. and Gagliano, W.B. (1998) Maternal control of embryogenesis by *MEDEA*, a polycomb-group gene in *Arabidopsis. Science.* **280**, 446-450.

Guscin, D. and Wolffe, A.P. (1999) Transcriptional control: SWItched on mobility. *Current Biology.* **9**, R742-R746.

Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1994) Regulation of the *Arabidopsis* floral homeotic gene *APETALA1. Cell,* **6**, 131-143.

Hagstrom, K. and Schedl, P. (1997) Remembrance of things past: maintaining gene expression patterns with altered chromatin. *Current Opinion in Genetics and Development,* **7**, 814-821.

Haig, D, and Westoby, M. (1991) Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its limitations for the evolution of apomixis. *Philos Trans R Soc London Ser B.* **333**, 1-13.

Hantke, S.S., Carpenter, R. and Coen, E.S. (1995) Expression of *floricaula* in single cell-layers of periclinal chimeras activates downstream homeotic genes in all layers of floral meristems. *Development.* **121**, 27-35.

Hauser, B.A., He, J.Q., Park, S.O. and Gasser, C.S. (2000) TSO1 is a novel protein that modulates cytokinesis and cell expansion in *Arabidopsis. Development.* **127**, 2219-2226.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42**, 819-832.

Holdeman, R., Nehrt, S. and Strome, S. (1998) MES-2 a maternal protein essential for viability of the germline in *Caenorhabditis elegans* is homologous to a *Drosophila* Polycomb group protein. *Development.* **125**, 2457-2467.

Honma, T. and Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature.* **409**, 525-529.

Huang, H., Mizukami, Y., Hu, Y. and Ma, H. (1993) Isolation and characterization of the binding sequences for the product of the *Arabidopsis* floral homeotic gene *AGAMOUS. Nucleic Acids Research.* **21**, 4769-4776.

Huijser, P., Klein, J., Lonnig, W-E., Meijer, H., Saedler, H. and Sommer, H. (1992) Bractomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *squamosa* in *Antirrhinum majus. The EMBO Journal* **11**, 1239-1249.

Ingram, G.C., Doyle, S., Carpenter, R., Schultz, E.A., Simon, R. and Coen, E.S. (1997) Dual roles for *fimbriata* in regulating floral homeotic genes and cell division in *Antirrhinum. The EMBO Journal* **16**, 6521-6534.

Ingram, G.C., Goodrich, J., Wilkinson, M.D., Simon, R., Haughn, G.W. and Coen, E.S. (1995) Parallels between *UNUSUAL FLORAL ORGANS* and *FIMBRIATA*,

genes controlling flower development in *Arabidopsis* and *Antirrhinum. The Plant Cell,* **7**, 1501-1510.

Irish, E.E. and Nelson, T.M. (1991) Identification of multiple stages in the conversion of maize meristems from vegetative to floral development. *Development.* **112**, 891-898.

Irish, V.F. and Sussex, I.M. (1990) Function of the *apetala-1* gene during *Arabidopsis* floral development. *The Plant Cell.* **2**, 741-753.

Ish-Horovitz, D. and Burke, J.F. (1981) Rapid and efficient cosmid cloning. *Nucleic Acids Research.* **9**, 2989-2995.

Ito, T., Takahashi, N., Shimura, Y. and Okada, K. (1997) A serine/threonine protein kinase gene isolated by an *in vivo* binding procedure using the *Arabidopsis* floral homeotic gene product, AGAMOUS. *Plant Cell Physiol.* **38**, 248-258.

Ivanova, A.V., Bonaduce, M.J., Ivanov, S.V. and Klar, A.J.S. (1998) The chromo and SET domains of the Clr4 protein are essential for silencing in fission yeast. *Nature Genetics.* **19**, 192-195.

Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994) *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and post transcriptional regulation determine floral organ identity. *Cell.* **76**, 703-716.

Jeddeloh, J.A., Stokes, T.L. and Richards, E.J. (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nature Genetics.* **22**, 94-97.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal.* **6**, 3901-3907.

Jenik, P.D. and Irish, V.F. (2000) Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. *Development.* **127**, 1267-1276.

Jofuku, D., den Boer, B., Van Montagu, M. and Okamuro, J. (1994). Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2. Plant Cell* **6**, 1211-1225.

Jones, C.A., Ng, J., Peterson, A.J., Morgan, K., Simon, J. and Jones, R.S. (1998) The *Drosophila* esc and E(z) proteins are direct partners in Polycomb Group-mediated repression. *Molecular and Cellular Biology.* **18**, 2825-2834.

Jones, D.A., Cowell, I.G. and Singh, P.B. (2000) Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays.* **22**, 124-127.

Jones, R.S. and Gelbart, W.M. (1990) Genetic analysis of the *Enhancer of zeste* locus and its role in gene regulation in *Drosophila melanogaster. Genetics.* **126**, 185-199.

Jones, R.S. and Gelbart, W.M. (1993) The *Drosophila* Polycomb-Group *Enhancer of zeste* contains a region with sequence similarity to *trithorax. Molecular and Cellular Biology.* **13**, 6357-6366.

Kassis, J.A. (1994) Unusual properties of regulatory DNA from the *Drosophila engrailed* gene: three "paired sensitive" sites within a 1.6kb region. *Genetics.* **136**, 1025-1038.

Kehle, J., Beuchle, D., Treuheit, S., Christen, B., Kennison, J.A., Bienz, M. and Muller, J. (1998) dMi-2, a hunchback-interacting protein that functions in *Polycomb* repression. *Science.* **282**, 1897-1899.

Kim, G-T, Tsukaya, H. and Uchimiya, H. (1998) The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana. Planta.* **206**, 175-183.

Kinoshita, T., Yadegari, R., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (1999) Imprinting of *MEDEA* Polycomb gene in the *Arabidopsis* endosperm. *The Plant Cell.* **11**, 1945-1952.

Kozak, M. (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *Journal of Molecular Biology.* **196**, 947-50.

Lacher, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature.* **410**, 116-120.

Laible, G., Wolf, A., Dorn, R., Reuter, G., Nislow, C., Lebersorger, A., Popkin, D., Pillus, L. and Jenuwein, T. (1997) Mammalian homologues of the Polycomb-group gene *Enhancer of zeste* mediate gene silencing in *Drosophila* heterochromatin and at *S.cerevisiae* telomeres. *The EMBO Journal.* **16**, 3219-3232.

LaJeunesse, D. and Shearn, A. (1996) *E(z):* a polycomb group gene or a trithorax group gene? *Development.* **122**, 2189-2197.

Lee, I., Wolfe, D.S., Nilsson, O. and Weigel, D. (1997) A *LEAFY* co-regulator encoded by *UNUSUAL FLORAL ORGANS. Current Biology.* **7**, 95-104.

Liu, Z. and Meyerowitz, E.M. (1995) *LEUNIG* regulates *AGAMOUS* expression in *Arabidopsis* flowers. *Development.* **121**, 975-991.

Liu, Z., Running, M.P. and Meyerowitz, E.M. (1997) TSO1 functions in cell division during *Arabidopsis* flower development. *Development.* **124**, 665-672.

Lloyd, A.M., Schena, M., Walbot, V. and Davies, R.W. (1994) Epidermal cell fate determination in *Arabidopsis*: patterns by a steroid-inducible regulator. *Science.* **266**, 436-439.

Luo, D., Carpenter, R., Copsey, L., Vincent, C., Clark, J. and Coen, E. (1999) Control of organ asymmetry in flowers of *Antirrhinum. Cell.* **99**, 367-376.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J. and Chaudury, A.M. (1999) Genes controlling fertilization-independent seed development in *Arabidopsis thaliana. Proceedings of the National Academy of Science (USA).* **96**, 296-301.

Luo, D., Carpenter, R., Vincent, C., Copsey, L. and Coen, E. (1996) Origins of floral asymmetry in *Antirrhinum. Nature.* **383**, 794-799.

MacFarlane, S.A. and Popovich, A.H. (2000) Efficient expression of foreign proteins in roots from tobravirus vectors. *Virology.* **267**, 29-35.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1992) Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature.* **360**, 273-277.

Mandel, M.A. and Yanofsky, M.F. (1995) A gene triggering flower formation in *Arabidopsis*. *Nature.* **377**, 522-524.

Marteinssen, R. and Irish, V. (1999) Copying out our ABCs the role of gene redundancy in interpreting genetic hierarchies. *Trends in Genetics.* **15**, 435-437.

McConnell, J.R., Emery, J., Eshad, Y., Bao, N, Bowman, J. and Barton, M.K. (2001) Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. *Nature.* **411**, 709-713.

McSteen, P.C.M. (1997) thesis, John Innes Institute, Norwich, UK.

McSteen, P.C.M., Vincent, C.A., Doyle, S., Carpenter, R. and Coen, E.S. (1998) Control of floral homeotic gene expression and organ morphogenesis in *Antirrhinum. Development.* **125**, 2359-2369.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F. and Schmidt, R.J. (1996) Diversification of *c*-function activity in maize flower development. *Science.* **274**, 1537-1540.

Meyerowitz, E.M., Bowman, J.L., Brockman, L.L., Drews, G.N., Jack, T., Sieburth, L.E. and Weigel, D. (1991) A genetic and molecular model for flower development in *Arabidopsis thaliana. Development.* (Suppl.) **1**, 157-167.

Mizukami, Y. and Ma, H. (1992) Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic *Arabidopsis* plants alters floral organ identity. *Cell.* **71**, 119-131.

Mizukami, Y. and Ma, H. (1997) Determination of *Arabidopsis* floral meristem identity by *AGAMOUS. The Plant Cell.* **9**, 393-408.

Motte, P. Saedler, H. and Schwarz-Sommer, Z. (1998) *STYLOSA* and *FISTULATA*: regulatory components of the homeotic control of *Antirrhinum* floral organogenesis. *Development.* **125**, 71-84.

Ng, J., Hart, C.M., Morgan, K. and Simon, J.A. (2000) A Drosophila ESC-E(Z) protein complex is distinct from other Polycomb group complexes and contains covalently modified ESC. *Molecular and Cellular Biology.* **20**, 3069-3078.

Nislow, C., Ray, E. and Pillus, L. (1997) *SET1*, A yeast member of the *Trithorax* family, functions in transcriptional silencing and diverse cellular processes. *Molecular Biology of the Cell.* **8**, 2421-2436.

Norman, C., Runswick, M., Pollock, R. and Treisman, R. (1988) Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-*fos* serum response element. *Cell.* **55**, 989-1003.

Ohad, N., Margossian, L., Hsu, Y-C., Williams, C., Repetti, P. and Fischer, R.L. (1996) A mutation that allows endosperm development without fertilization. *Proceedings of the National Academy of Science (USA).* **93**, 5319-5324.

Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Micheali, D., Harada, J.J., Goldberg, R.B. and Fischer, R.L. (1999) Mutations in *FIE*, a WD Polycomb Group gene, allow endosperm development without fertilization. *The Plant Cell.* **11**, 407-415.

Okamuro, J.K., Caster, B., Villarroel, R., Van Montagu, M. and Jofuku, K.D. (1997) The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in *Arabidopsis. Proceedings of the National Academy of Science (USA).* **94**, 7076-7081.

Olson, E.N., Perry, M., Schulz, R.A. (1995) Regulation of muscle differentiation by the MEF2 family of transcription factors. *Developmental Biology.* **172**, 2-14.

Orlando, V., Jane, E.S., Chinwalla, V., Harte, P.J. and Paro, R. (1988) Binding of the Trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early *Drosophila* embryogenesis. *The EMBO Journal.* **17**, 5141-5150.

Parcy, F., Nilsson, O., Busch, M.A., Lee, I. and Weigel, D. (1998) A genetic framework for floral patterning. *Nature.* **395**, 561-566.

Passmore, S., Maine, G.T., Elble, R., Christ, C., and Tye, B.K. (1988) A *Saccharomyces cerevisiae* protein involved in plasmid maintenance is necessary for mating in MATα cells. *Journal of Molecular Biology.* **204**, 593-606.

Paro, R. (1995) Propagating memory of transcriptional states. *TIG.* **11**, 295-297.

Phillips, M.D. and Shearn, A. (1990) Mutations in *polycombeotic*, a *Drosophila* Polycomb-group gene, cause a wide range of maternal and zygotic phenotypes. *Genetics.* **125**, 91-101.

Pirrotta, V. (1997) PcG complexes and chromatin silencing. *Current Opinion in Genetics and Development.* **7**, 249-258.

Pirrotta, V. (1998) Polycombing the genome: PcG, trxG, and Chromatin silencing. *Cell.* **93**, 333-336.

Platero, J.S., Hartnett, T. and Eissenberg, J.C. (1995) Functional analysis of the chromo domain of HP1. *The EMBO Journal.* **14**, 3977-3986.

Poux, S., Kostic, C. and Pirrotta, V. (1996) Hunchback-independent silencing of late *Ubx* enhancers by a Polycomb Group Response Element. *The EMBO Journal.* **15**, 4713-4722.

Poux, S., McCabe, D. and Pirrotta, V. (2001) Recruitment of components of Polycomb Group chromatin complexes in *Drosophila*. *Development.* **128**, 75-85.

Rastelli, L., Chan, C.S. and Pirrotta, V. (1993) Related chromosome binding sites for *zeste*, suppressors of *zeste* and *Polycomb* group proteins in *Drosophila* and their dependence on *Enhancer of zeste* function. *The EMBO Journal.* **12**, 1513-1522.

Ratcliffe, O.J., Bradley, D.J. and Coen, E.S. (1999) Separation of shoot and floral identity in *Arabidopsis*. *Development.* **126**, 1109-1120.

Ratcliffe, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Tobacco rattle virus as a vector for the analysis of gene function by silencing. *The Plant Journal.* **25**, 237-245.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z-W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D. and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature.* **406**, 593-599.

Ringrose, L. and Paro, R. (2000) Remembering silence. *Bioessays.* **23.7**, 566-570.

Rosenblatt-Rosen, O., Rozovskaia, T., Burakov, D., Sedkov, Y., Tillib, S., Blechman, J., Nakamura, T., Croce, C.M., Mazo, A. and Canaani, E. (1998) The C-terminal SET domains of ALL-1 and TRITHORAX interact with the INI1 and SNR1 proteins, components of the SWI/SNF complex. *Proceedings of the National Academy of Science (USA).* **95**, 4152-4157.

Ruiz, M.T., Voinnet, O. and Baulcombe, D.C. (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell.* **10**, 937-946

Sablowski, R.W.M. and Meyerowitz, E.M. (1998) A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. *Cell.* **92**, 93-103.

Sakai, H., Medrano, L.J. and Meyerowitz, E.M. (1995) Role of *SUPERMAN* maintaining *Arabidopsis* floral whorl boundaries. *Nature.* **378**, 199-203.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S, Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis. Science.* **288**, 1613-1616.

Schumacher, A., Lichtarge, O., Schwartz, S. and Magnuson, T. (1998) The murine Polycomb-Group gene *eed* and its human orthologue: functional implications of evolutionary conservation. *Genomics.* **54**, 79-88.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.-E., Saedler, H. and Sommer, H. (1992) Characterization of the *Antirrhinum* homeotic MADS-box gene *deficiens*: Evidence of DNA binding and autoregulation of its persistent expression throughout flower development. *The EMBO Journal.* **11**, 251-263.

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. and Sommer, H. (1990) Genetic control of flower development by homeotic genes in *Antirrhinum majus. Science.* **250**, 931-936.

Scott, R.J., Speilman, M., Bailey, J. and Dickinson, H.G. (1998) Parent-of-origin effects on seed development in *Arabidopsis thaliana. Development.* **125**, 3329-3341.

Sessions, A., Yanofsky, M.F. and Weigel, D. (2000) Cell-cell signalling and movement by the floral transcription factors LEAFY and APETALA1. *Science.* **289**, 779-781.

Sewalt, R.G.A.B., van der Vlag, J., Gunster, M.J., Hamer, K.M., den Blaauwen, J.L., Satijn, D.P.E., Hendrix, T., van Driel, R. and Otte, A.P. (1998) Characterization of interactions between the mammalian Polycomb-Group proteins Enx1/EZH2 and EED suggests the existence of different mammalian Polycomb-Group protein complexes. *Molecular and Cellular Biology.* **18**, 3586-3595.

Seyoux, G. and Strome, S. (1999) Launching the germline in *Caenorhabditis*: regulation of gene expression in early germ cells. *Development.* **126**, 3275-3283.

Schumacher, A., Faust, C. and Magnuson, T. (1996) Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature.* **383**, 250-253.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C-T., Bender, W. and Kingston, R.E. (1999) Stabilisation of chromatin structure by PRC1, a Polycomb complex. *Cell.* **98**, 37-46.

Shearn, A., Hersperger, G., and Hersperger, E. (1978) Genetic analysis of two allelic temperature-sensitive mutants of *Drosophila melanogaster* both of which are zygotic and maternal-effect lethals. *Genetics.* **89**, 341-353.

Sieburth, L.E., Drews, G.N. and Meyerowitz, E.M. (1998) Non-autonomy of *AGAMOUS* function in flower development: use of a *Cre/loxP* method for mosaic analysis in *Arabidopsis. Development.* **125**, 4303-4312.

Sieburth, L.E. and Meyerowitz E.M. (1997) Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *The Plant Cell.* **9**, 355-365.

Simon, J., Bornemann, D., Lunde, K. and Schwartz, C. (1995) The *extra sex combs* product contains WD40 repeats and its time of action implies a role distinct from other *Polycomb* group products. *Mechanisms of Development.* **53**, 197-208.

Simon, J., Chiang, A. and Bender, W. (1992) Ten different *Polycomb* group genes are required for spatial control of the *abdA* and *AdbB* homeotic products. *Development.* **114**, 493-505.

Simon, R., Igeno, M.I. and Coupland, G. (1996) Activation of floral meristem identity genes in *Arabidopsis. Nature.* **384**, 59-62.

Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *The Plant Cell.* **2**, 755-767.

Sommer, H., Beltran, J-P., Huijser, P., Pape, H., Lonnig, W-E., Saedler, H. and Schwarz-Sommer, Z. (1990) *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. *The EMBO Journal.* **9**, 605-613.

Sondex, J., Bohm, A, Lambright, G., Hamm, H.E. and Sigler, P.B. (1996) Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1A resolution. *Nature.* **379**, 369-374.

Song, J.Y., Leung, T., Ehler, L.K., Wang, C. and Liu, Z. (2000) Regulation of meristem organisation and cell division by TSO1, an *Arabidopsis* gene with cysteine-rich repeats. *Development.* **127**, 2207-2217.

Sorensen, M.B., Chaudury, A.M., Robert, H., Bancharel, E. and Berger, F. (2001) Polycomb group genes control pattern formation in plant seed. *Current Biology.* **11**, 277-281.

Spillane, C., MacDougall, C., Stock, C., Kohler, C., Vielle-Calzada, J-P., Nunes, S.M., Grossniklaus, U. and Goodrich, J. (2000) Interaction of *Arabidopsis* Polycomb group proteins FIE and MEA mediates their common phenotypes. *Current Biology.* **10**, 1535-1538.

Stassen, M.J., Bailey, D., Nelson, S., Chinwalla, V. and Harte, P.J. (1995) The *Drosophila trithorax* protein contains a novel variant of the nuclear receptor type DNA binding domain and an ancient conserved motif found in other chromosomal proteins. *Mechanisms of Development.* **52**, 209-223.

Struhl, G. (1981) A gene product required for correct initiation of segmental determination in *Drosophila. Nature.* **293**, 36-41.

Struhl, G. (1982) Genes controlling segmental specification in the Drosophila thorax. *Proceedings of the National Academy of Science (USA).* **79**, 7380-7384.

Struhl, G. and Brower, D. (1982) Early role of the esc+ gene product in the determination of segments in *Drosophila. Cell.* **31**, 285-292.

Stubbe, H. (1966) *Genetik und Zytologie von Antirrhinum l. sec. Antirrhinum.* VEB Gustav Fisher, Jena, German Democratic Republic.

Strutt, H., Cavalli, G. and Paro, R. (1997) Co-localisation of *Polycomb* protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *The EMBO Journal.* **16**, 3621-3632.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) Brahma: a regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell.* **68**, 561-572.

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J.T., Munster, T, Winter, K-U. and Saedler, H. (2000) A short history of MADS-box genes in plants. *Plant Molecular Biology.* **42**, 115-149.

Tie, F., Furuyama, T. and Harte, P.J. (1998) The *Drosophila* Polycomb Group proteins ESC and E(z) bind directly to each other and co-localize at multiple chromosomal sites. *Development.* **125**, 3483-3496.

Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E. and Harte, P.J. (2001) The Drosophila Polycomb Group proteins ESC and E(z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. *Development.* **128**, 275-286.

Trobner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W-E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1992) *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *The EMBO Journal.* **11**, 4693-4704.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G. and Reuter, G. (1994) The protein encoded by the *Drosophila* position-effect variegation suppressor gene *Su(var)3-9* combines domains of antagonistic regulators of homeotic gene complexes. *The EMBO Journal.* **13**, 3822-3831.

Van der Vlag, J. and Otte, A.P. (1999) Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nature Genetics.* **23**, 474-478.

van Lohuizen, M. (1999) The trithorax and Polycomb-group chromatin modifiers: implications for disease. *Current Opinion in Genetics and Development.* **9**, 355-361.

van Lohuizen, M., Tijms, M., Voncken, J.W., Schumacher, A., Magnuson, T. and Wientjens, E. (1998) Interaction of mouse Polycomb-Group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes. *Molecular and Cellular Biology.* **18**, 3572-3579.

Vielle-Calzada, J-P., Thomas, J., Spillane, C., Coluccio, A., Hoeppner, M.A. and Grossniklaus, U. (1999) Maintenance of genomic imprinting at the *Arabidopsis medea* locus requires zygotic *DDM1* activity. *Genes and Development.* **13**, 2971-2982.

Vincent, C.A., Carpenter, R. and Coen, E.S. (1995) Cell lineage patterns and homeotic gene activity during *Antirrhinum* flower development. *Current Biology.* **5**, 1449-1458.

Voinnet, O., Lederer, C. and Baulcombe, D.C. (2000) A viral movement protein prevents systemic spread of the gene silencing signal. *Cell.* **103**, 157-167.

Wagner, D., Sablowski, R.W.M. and Meyerowitz, E.M. (1999) Transcriptional activation of APETALA 1 by LEAFY. *Science.* **285**, 582-584.

Waites, R., Selvadurai, H.R.N., Oliver, I.R. and Hudson, A (1998) The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum. Cell.* **93**, 779-789.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F. and Meyerowitz, E.M. (1992) *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell.* **69**, 843-859.

Weigel, D. and Doerner, P. (1996) Cell-cell interactions: taking cues from the neighbours. *Current Biology.* **6**, 10-12.

Weigel, D. and Meyerowitz, E.M. (1993) Activation of floral homeotic genes in *Arabidopsis*. *Science.* **261**, 1723-1726.

Weigel, D. and Nilsson, O. (1995) A developmental switch sufficient for flower initiation in diverse plants. *Nature.* **377**, 495-500.

Williams, L. and Grafi, G. (2000) The retinoblastoma protein-a bridge to heterochromatin. *Trends in Plant Science.* **5**, 239-240.

Xie, Q., Sanz-Burgos, P., Hannon, G.J. and Gutierrez, C. (1996) Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. *The EMBO Journal.* **15**, 4900-4908.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature.* **346**, 35-39.

Zachgo, S., de Andrade Silva, E., Motte, P., Trobner, W., Saedler, H. and Schwarz-Sommer, Z. (1995) Functional analysis of the *Antirrhinum* floral homeotic *DEFICIENS* gene in vivo and in vitro by using a temperature-sensitive mutant. *Development.* **121**, 2861-2875.

Zhang, C-C., Bienz, M. (1992) Segmental determination in *Drosophila* conferred by *hunchback,* a direct repressor of the homeotic gene *Ultrabithorax. Proceedings of the National Academy of Science (USA).* **89**, 7511-7515.

Zink, D. and Paro, R. (1995) *Drosophila* Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. *The EMBO Journal.* **14**, 5660-5671.